EXPLORING CLINICAL AND GENETIC FACTORS TO OPTIMIZE THE USE OF TYROSINE KINASE INHIBITORS IN CLINICAL PRACTICE

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ABSTRACT

Nancy K. Gillis: Exploring Clinical and Genetic Factors to Optimize the Use of Tyrosine Kinase Inhibitors in Clinical Practice
(Under the direction of Howard L. McLeod and Tim Wiltshire)

Multi-targeted tyrosine kinase inhibitors (TKIs) are widely prescribed anticancer agents that provide significant benefit in survival across a range of cancers; however, 20-30% of individuals do not respond, demonstrating intrinsic resistance. The goal of this dissertation was to identify demographic, clinical, and genetic factors predictive of inefficacy to the multi-targeted TKIs.

A retrospective analysis of 108,825 cancer patients revealed a heightened incidence of a Stevens-Johnson syndrome (SJS), a life-threatening adverse event, in cancer patients and suggested that TKIs may trigger the reaction, necessitating drug discontinuation. In a retrospective study of outcomes in routine clinical practice, chart reviews of 266 patients treated with multi-targeted TKIs identified a resistance rate of 21%. Duration of TKI treatment was significantly longer in non-resistant patients; however, there were no significant differences in demographics, tumor type treated, or TKI received. Similar rates of resistance in the subgroups suggest that there may be unidentified shared markers of resistance to these agents.

A methodology study to determine the utility of archived formalin-fixed paraffin-embedded (FFPE) samples for genetic analyses was conducted. FFPE tumor samples representing a range of ages, tissue sources, and diagnoses were obtained for next-generation sequencing (NGS). We found no association between age of FFPE samples and sequencing failure; however, there was an association between DNA yield and ability to generate NGS. Both

failed samples were derived from bone, suggesting that bone is not an ideal source for FFPE-derived DNA.

To identify genetic predictors of intrinsic resistance, cancer patients treated with multi-targeted TKIs were classified as resistant or non-resistant, and tumor FFPE samples were used to generate NGS and copy number variation (CNV). *NTRK1*, *KDR*, *TGFBR2*, and *PTPN11* were more commonly mutated in resistant patients and *CDK4*, *CDKN2B*, and *ERBB2* demonstrated differential patterns of CNVs between groups. In combined analysis using random forest and a decision tree, CNV in *CDK4* and *CDKN2B* were the most important features, and alone could differentiate 55% of individuals as resistant or non-resistant, thus implicating the cyclin-dependent pathway as an important factor in resistance to multi-targeted TKIs. This dissertation presents foundational evidence for the personalization of multi-targeted TKI prescribing and management.

To My Loving Family and Friends

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LIST OF ABBREVIATIONS

A	Adenine
ABCB1	ATP binding cassette subfamily B member 1
ABCG2	ATP binding cassette subfamily G member 2
ABL1	Abelson murine leukemia viral oncogene, homolog 1
AIDS	Acquired immune deficiency syndrome
AKT	Protein kinase B alpha
ALK	Anaplastic lymphoma kinase
ALL	Acute lymphocytic leukemia
AML	Acute myeloid leukemia
ARAF	A-Raf proto-oncogene
AXL	AXL receptor tyrosine kinase
BAF	Minor (B) allele frequency
BCL2	B-cell CLL/lymphoma 2
BCR-ABL	Breakpoint cluster region- Abelson tyrosine-protein kinase 1 fusion gene
BCRP	Breast cancer resistance protein
BH3	Bcl-2 homology domain 3
BIM	Bcl-2 interacting mediator of cell death, BCL2-like 11
BMX	BTK-like on X chromosome
BRAF	B-raf proto-oncogene
BTK	Bruton tyrosine kinase
BWA	Burrows-Wheeler Aligner
C	Cytosine
CCND1	Cyclin D1

CDK12	Cyclin dependent kinase 12
CDK4	Cyclin dependent kinase 4
CDKN2B	Cyclin dependent kinase inhibitor 2B
CI	Confidence interval
c-KIT	C-KIT proto-oncogene receptor tyrosine kinase
CML	Chronic myeloid leukemia
CNV	Copy number variation
COSMIC	Catalogue of Somatic Mutations in Cancer
COT	Cancer Osaka thyroid oncogene, *MAP3K8*
CRAF	C-Raf proto-oncogene
CRC	Colorectal carcinoma
CSF1	Colony stimulating factor 1
CT	Computerized tomography
Cyclin D	Cyclin-dependent
CYP3A5	Cytochrome P450 3A5
DDR	Discoidin domain receptor
DIN	DNA integrity number
DNA	Deoxyribonucleic acid
EGFR	Epidermal growth factor receptor
EML4	Echinoderm microtubule associated protein like 4
EMT	Epithelial to mesenchymal subtype
EPHA3	EPH Receptor A3
ERBB2	Erb-B2 receptor tyrosine kinase 2, encodes HER2

ERBB3	Erb-B2 receptor tyrosine kinase 3, encodes HER3
ERK	Extracellular signal-regulated kinase
ESR1	Estrogen receptor 1
FDA	U.S. Food and Drug Administration
FDR	False-discovery rate
FF	Fresh frozen
FFPE	Formalin-fixed paraffin-embedded
FGFR	Fibroblast growth factor receptor
FGFR1	Fibroblast growth factor receptor 1
FGFR4	Fibroblast growth factor receptor 4
FLT3	Fms related tyrosine kinase 3
G	Guanine
GATK	Genome Analysis ToolKit
GIST	Gastrointestinal stromal tumor
GQ	Genotype quality
H&E	Haematoxylin and eosin (staining)
HCC	Hepatocellular carcinoma
HER2	Human epidermal growth factor receptor 2
HER3	Human epidermal growth factor receptor 3
HER4	Human epidermal growth factor receptor 4
HGF	Hepatocyte growth factor
HIV	Human immunodeficiency virus
HLA	Human leukocyte antigen, encodes the major histocompatibility complex

HPRT	Hypoxanthine phosphoribosyltransferase
hr.	Hour
HR	Hazard ratio
HRAS	Harvey rat sarcoma viral oncogene homolog
HSP	Heat shock protein
HSP90	Heat shock protein 90
HT1	Hybridization buffer
IC_{50}	Half maximal inhibitory concentration
ICD-9	International Classification of Disease, version 9
IGF-1R	Insulin-like growth factor 1 receptor
IRB	Institutional review board
JMML	Juvenile myelomonocytic leukemia
KDR	Kinase insert domain receptor
KIT	V-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene
KRAS	Kirsten rat sarcoma viral oncogene
LRP1B	LDL Receptor Related Protein 1B
MALDI-MSI	Matrix-assisted laser desorption ionization mass spectrometry imaging
Mapk14-Atf2	Mitogen-activated protein kinase 14-Activating transcription factor 2
MAP2K1	Mitogen-activated protein kinase kinase 1, *MEK1*
MAP3K8	Mitogen-activated protein kinase kinase kinase 8, *COT*
MAPD	Median absolute pairwise difference
MAPK	Mitogen-activated protein kinase
Mb	Megabase

MCC	Moffitt Cancer Center
MCL	Mantle cell lymphoma
MDS	Myelodysplastic syndrome
MEK	Mitogen-activated protein kinase kinase enzyme
MEK1	Mitogen-activated protein kinase kinase enzyme 1, *MAP2K1*
MEK2	Mitogen-activated protein kinase kinase enzyme 2, *MAP2K2*
MET	MET proto-oncogene
μg	Microgram
μL	Microliter
μm	Micromole
min	Minute
MLL	Myeloid/lymphoid or mixed-lineage leukemia, *KMT2A*
mo.	Month
MRI	Magnetic resonance imaging
MRN	Medical record number
mTOR	Mechanistic target of rapamycin
NA	Not applicable
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
ndSNPQC	Normal diploid SNP quality control
NF1	Neurofibromin 1
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
ng	Nanogram

NGS	Next-generation sequencing
NHLBI	National Heart, Lung, and Blood Institute
nM	Nanomolar
NRAS	Neuroblastoma RAS viral oncogene homolog
NSCLC	Non-small cell lung cancer
NTRK	Neurotrophic receptor tyrosine kinase
NTRK1	Neurotrophic receptor tyrosine kinase 1
OR	Odds ratio
ORR	Overall response rate
OS	Overall survival
PCR	Polymerase chain reaction
PDGFR	Platelet-derived growth factor receptor
PDGFRA	Platelet-derived growth factor receptor alpha
PDGFRB	Platelet-derived growth factor receptor beta
PET	Positron emission tomography
PFS	Progression free survival
P-gp	Permeability glycoprotein
Ph	Philadelphia chromosome
PHI	Protected health information
PHLPP1	PH domain and leucine rich repeat protein phosphatase 1
PI3K	Phosphatidylinositol 3-kinase
PIK3CA	Phosphatidylinositol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3CG	Phosphatidylinositol-4,5- bisphosphate 3-kinase catalytic subunit gamma

PIK3R2	Phosphoinositide-3-kinase regulatory subunit 2
PKI	Protein kinase inhibitor
PLCg2	Phospholipase Cy2
pM	Picomolar
pNET	Pancreatic neuroendocrine tumor
PTEN	Phosphatase and tensin homolog
PTPN11	Protein tyrosine phosphatase, non-receptor type 11
QC	Quality control
qPCR	Quantitative polymerase chain reaction
RAC1	Ras-related C3 botulinum toxin substrate 1
RAF	Rapidly accelerated fibrosarcoma
RAS	Rat sarcoma viral oncogene
RCC	Renal cell carcinoma
RET	Ret proto-oncogene
RNA	Ribonucleic acid
ROS	ROS proto-oncogene
SCLC	Small cell lung cancer
SEGA	Subependymal giant cell astrocytoma
SHP2	Src homology-2 domain-containing phosphatase 2, encoded by *PTPN11*
SJS	Stevens-Johnson syndrome
SMO	Smoothened, frizzled class receptor
SRC	SRC proto-oncogene
STAT3	Signal transducer and activator of transcription 3

T	Thymine
TCC	Total Cancer Care
TCGA	The Cancer Genome Atlas
TEK	TEK receptor tyrosine kinase (endothelial tyrosine kinase), *TIE2*
TIE2	TEK receptor tyrosine kinase (endothelial tyrosine kinase), *TEK*
TEN	Toxic epidermal necrolysis
TGFBR2	Transforming growth factor beta receptor 2
TKI	Tyrosine kinase inhibitor
TNFAIP3	Tumor necrosis factor, alpha-induced protein 3
TPMT	Thiopurine S-methyltransferase
TSC1	Tuberous sclerosis 1
TSC2	Tuberous sclerosis 2
Ts/Tv	Transition-to-transversion ratio
U.S.	United States
VEGF	Vascular endothelial growth factor
VEGFR1	Vascular endothelial growth receptor 1, encoded by *FLT1*
VEGFR2	Vascular endothelial growth receptor 2, encoded by *KDR*
VEGFR3	Vascular endothelial growth receptor 3, encoded by *FLT4*
VQSR	Variant quality score recalibration
VS.	Versus
WES	Whole exome sequencing
WM	Waldenstrom macroglobulinemia
YAP	Yes-associated protein

YAP1	Yes-associated protein 1
yr	Year

CHAPTER 1: BACKGROUND: THE PHARMACOGENOMICS OF DRUG RESISTANCE TO PROTEIN KINASE INHIBITORS[1]

Summary

Dysregulation of growth factor cell signaling is a major driver of most human cancers. This has led to the development of numerous drugs targeting protein kinases, with demonstrated efficacy in the treatment of a wide spectrum of cancers. Despite their high initial response rates and survival benefits, the majority of patients eventually develop resistance to these targeted therapies. Discussed in this chapter are examples of established mechanisms of drug resistance to anticancer therapies, including drug target mutations or gene amplifications, emergence of alternate signaling pathways, and pharmacokinetic variation. This reveals a role for pharmacogenomic analysis to identify and monitor for resistance, with possible therapeutic strategies to combat resistance. Gaps in the understanding of mechanisms of intrinsic resistance are also highlighted.

Introduction

Cancer is a genetic disease that arises primarily from the accumulation of genetic changes in genes regulating cellular growth, proliferation, and survival. Gain of function alterations inducing hyperactivity of oncogenes or loss of function alterations leading to inactivation of tumor suppressor genes cause deregulation of cellular signaling, a fundamental trait of cancer

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cells. In healthy cells, homeostasis is conveyed via growth factors binding to cell surface receptors, primarily protein kinases, which then activate intracellular signaling pathways and regulate cell cycle progression.[1] Deregulation of these signals results in uncontrollable cellular proliferation, metabolism, survival and, ultimately, cancer. Somatic (acquired, or tumor) mutations lead to constitutive activation of these signaling pathways. For example, mutations in the B-raf proto-oncogene, *BRAF*, a serine/threonine kinase, cause constitutive signaling through the mitogen-activated protein kinase (MAPK) pathway and are commonly observed in melanoma, colorectal (CRC), and papillary thyroid cancers.[2] Identification of the genes and pathways deregulated in cancer, such as *BRAF*, has led to a rapid increase in the design and approval of therapies targeting these genetic drivers of oncogenesis. Targeted anti-cancer therapies function by interfering with specific molecular alterations that regulate cellular signaling and drive tumor growth. By binding to or inhibiting a known molecular driver, targeted therapies interrupt the signaling pathways, causing cellular deregulation and leading to cancer cell apoptosis or cell death. For example, vemurafenib selectively inhibits *BRAF V600*-mutated cancer cells, abrogating MAPK-mediated signaling, preventing proliferation of *BRAF*-mutated cells, and ultimately resulting in apoptosis.[3]

To optimize the use of targeted therapies, the genetic alterations causing pathway deregulation in each patient's tumor must be identified. This is the modern concept of personalized cancer medicine (or precision medicine). Pharmacogenomics is the study of how genetic variations influence the response of an individual to drugs. In the context of cancer, there are two genomes relevant to predicting drug response or resistance: (1) germline, or inherited, genetics may affect drug exposure, potentially causing variability in efficacy and/or toxicity, and (2) somatic, or tumor, genetics are the acquired alterations that may initiate and perpetuate

cellular deregulation. Generally, it is the somatic germline that is interrogated to identify alterations driving oncogenesis and to select targeted therapies. Despite overall efficacy of targeted agents, drug resistance phenomena continue to be a primary hindrance to the curative treatment of solid tumors and hematologic malignancies.[4-6] Hence, deciphering the molecular mechanisms underlying resistance should enhance targeted individualized cancer medicine.[7-9]

Due to their critical role in regulating cellular signaling, greater than twenty protein kinase inhibitors (PKIs) have been developed and approved across a wide range of tumor types. Despite their overall high response rates, many patients for whom the drugs are indicated will not have any evidence of disease control, while others will have transient benefit followed by tumor growth. This lack of complete and durable responses is indicative of drug resistance. If the correct alteration driving tumor growth is not identified from the outset, intrinsic resistance may be observed. On the other hand, when the tumor cells evolve mechanisms to overcome targeted inhibition, the patient develops acquired resistance and stops responding to therapies that were previously effective. Discussed in this chapter are the various classifications of cancer drug resistance, examples of how pharmacogenomics plays a role in resistance to PKIs, and possible therapeutic strategies to overcome cancer drug resistance.

Classifications of drug resistance mechanisms important in cancer

Drug resistance is the lack of therapeutic benefit or response to a medication. In cancer, drug resistance is apparent with an increase in tumor size or metastasis (i.e., disease progression). Various mechanisms of resistance can result in lack of complete or durable response to cancer therapies (Figure 1.1). An overview of the common classifications of resistance is provided below. It is important to note that a single pharmacogenomic biomarker may represent multiple mechanisms of drug resistance.

Pharmacological vs. biological resistance

Pharmacological resistance reflects inadequate drug exposure at the drug target, and can be caused by environmental factors (e.g., drug-drug interactions, non-compliance), germline pharmacogenomics (i.e., inter-individual variability in drug metabolism or pharmacokinetics) (Figure 1.1A & 1.1C), or drug sequestration away from its target.[10,11] For example, addition of an antacid to alleviate gastroesophageal reflux caused by some PKI therapies will affect gastric absorption and exposure to the PKI, thereby leading to pharmacological resistance.[12] The observation that PKI-sensitive clones reemerge post-PKI discontinuation demonstrates the cytostatic, rather than cytotoxic, nature of some targeted therapies.[13,14] Therefore, inconsistent suppression of the drug target due to missed doses may lead to upregulation of the cancer-driving pathways and, ultimately, cancer progression.

Biological resistance results from cancer cell evolution in the presence of adequate drug exposure.[5,6,15] In the context of cancer, biological resistance can arise from somatic alterations in drug targets or pathways (Figure 1.1B & 1.1C). Examples include genetic alterations in the drug target itself, activation of alternative signaling pathways (bypass tracks), alteration in signaling proteins downstream of the drug target, or phenotypic switch. Most known mechanisms of resistance to targeted cancer therapies are of the biological resistance subtype.

Intrinsic vs. acquired resistance

Cancer drug resistance can also be classified based on timing during the course of treatment. Intrinsic resistance (also referred to as innate, inherent, or primary resistance) is the absence of discernible, even transitory beneficial effect from a medication. From the outset, there is neither cessation in tumor growth nor increase in survival benefits (Figure 1.1D). Evidence of intrinsic resistance is apparent in waterfall plots, in which some patients fail to meet Response

Evaluation Criteria in Solid Tumors. For example, approximately 20% of *BRAF V600*-mutated melanoma patients do not respond to BRAF inhibitors, demonstrating intrinsic drug resistance.[16] Mechanisms of intrinsic resistance can include germline or somatic alterations. Intrinsic resistance is a major challenge in cancer therapy, and it is important to elucidate the mechanisms conferring lack of therapeutic benefit; however, these mechanisms remain less well understood at this time.

Acquired or secondary resistance is the progression of disease after an initial benefit. In oncology, the tumor initially shrinks (responds) but eventually begins to increase in size (Figure 1.1E). While intrinsic drug resistance can be due to germline or somatic mutations, acquired resistance is most commonly attributed to somatic mutations (an exception would be a new drug-drug interaction due to changes in therapy). Mechanisms that result in acquired drug resistance include genetic alterations in the drug target, activation of bypass tracks, alteration in downstream signaling proteins, phenotypic switch, drug efflux, and drug compartmentalization.[15,17,18]

Germline pharmacogenomics as a mechanism of pharmacological resistance

Germline pharmacogenomics can affect one's inherent response to a medication or therapy. In the context of cancer, germline pharmacogenomics has most widely been associated with risk of developing adverse effects, rather than drug resistance.[19] Since adverse effects generally result from off-target (i.e., non-tumor) effects, it is logical that genetic variation in the germ cells throughout the body would confer risk to adverse events. However, due to the inherited nature of germline genetics, they may also play a role in one's initial response to therapy. In fact, there are a few well-studied examples of how germline variation can confer intrinsic resistance to anticancer medications (Table 1.1).

Germline variation in TPMT as a pharmacogenomics predictor of response and adverse events

Germline variation in *TPMT*, the gene that encodes the thiopurine S-methyltranserfase (TPMT) enzyme, affects response to thiopurine drugs. Chemotherapeutic thiopurines include 6-mercaptopurine and 6-thioguanine, which are used in the treatment of pediatric and adult acute lymphocytic leukemia (ALL) (azathioprine is a prodrug of 6-mercaptopurine used in non-malignant conditions). Thiopurine drugs are inactive prodrugs that are bioactivated and metabolized via competing routes: (1) xanthine oxidase converts 6-mercaptopurine to an inactive metabolite, 6-thiouric acid; (2) hypoxanthine phosphoribosyltransferase (HPRT) converts thiopurines to activated nucleotide analogues, which can be incorporated into DNA or RNA, hence interfering with replication and transcription, resulting in cytotoxicity; and (3) TPMT inactivates thiopurines through methylation. The nucleotide analogues formed by HPRT are responsible for the efficacy and toxicity of thiopurine drugs, and insufficient TPMT activity results in upregulation of HPRT-mediated metabolism, conferring response. Patients with low TPMT activity have greater exposure to activated thioguanine nucleotides, resulting in the potential for greater efficacy, but also increased risk of severe toxicity.[20] This variation in activated nucleotide exposure is a well-established predictor of treatment outcomes. Patients with *TPMT* loss-of-function variants have significantly lower rates of minimal residual disease positivity after receiving 6-mercaptopurine therapy when compared to wild-type *TPMT* individuals.[21] Increased risk of relapse has also been associated with wild-type *TPMT* in children receiving 6-mercaptopurine, likely due to insufficient thioguanine nucleotide exposure, thus conferring a type of pharmacological resistance.[22] Due to increased toxicity risk, decreased dosing for patients with *TPMT* variants is recommended;[23] however, it is unclear how this may affect relapse rates.[24,25] A recent study reported that patients with 6-mercaptopurine non-

adherence were at a 2.7-fold increased risk of relapse when compared to patients with a mean drug adherence rate of 95% or greater ($p = 0.01$), further emphasizing the importance of continuous drug exposure and adherence as a means to avoid development of drug resistance.[26]

Germline alterations in BIM as a predictor of intrinsic pharmacological resistance

A common variant in *BCL2L11* (also known as *BIM*) has been associated with intrinsic resistance to PKIs. *BIM* is a member of the B-cell CLL/lymphoma 2 (Bcl-2) family of genes and encodes a Bcl-2 homology domain 3 (BH3). BH3 activates cell death by either opposing the pro-survival members of the Bcl-2 family or by binding to the pro-apoptotic Bcl-2 family members and causing activation of their pro-apoptotic functions.[27] PKIs induce upregulation and stabilization of BIM through inhibition of the MAPK pathway, therefore, the activity of BIM is required for PKIs to induce apoptosis in kinase-driven cancers.[28] Recently, a 2,903 bp germline deletion polymorphism in intron 2 of *BIM* was identified, which was associated with inferior responses to PKIs (i.e., imatinib, gefitinib, erlotinib, and afatinib) in chronic myeloid leukemia (CML), non-small cell lung cancer (NSCLC), and pediatric ALL patients.[29-31] Functionally, this mutation results in alternative RNA splicing, leading to decreased production of BIM isoforms containing the essential BH3 domain.

Since its discovery, conflicting evidence of the ability of *BIM* variation to predict intrinsic resistance to PKIs has been reported.[32-34] Two retrospective studies failed to observe an association between *BIM* genotype and response rates to PKIs in NSCLC patients.[35,36] However, a systematic review and meta-analysis of 951 patients supported the *BIM* deletion polymorphism as a predictor of shorter progression free survival (PFS) in NSCLC patients who were treated with PKIs (adjusted HR = 2.38, $p < 0.001$).[37] Another meta-analysis found that the *BIM* deletion polymorphism was associated with response rates (HR = 0.44, 95% CI = 0.27-0.7) and PFS (HR

= 2.19, 95% CI = 1.7-2.8) in NSCLC, but not in CML.[38] Further evidence indicating a lack of benefit or increased risk of harm in individuals carrying *BIM* deletions is warranted before this biomarker of intrinsic resistance can be implemented in clinical practice.

Methods to overcome BIM-related PKI resistance are already being explored. A preclinical study in NSCLC cell lines and xenograft models indicated that cells harboring the common *BIM* deletion had enhanced response to gefitinib when treated in combination with a histone deacetylase inhibitor, vorinostat.[39] Vorinostat functioned by increasing expression of BH3 in a dose-dependent manner, thus restoring sensitivity to tyrosine kinase inhibition. These findings further support the importance of *BIM* expression in PKI response and provide evidence to suggest that combination therapeutics may be a potential strategy to overcome this form of resistance.

Additional germline pharmacogenomic markers as predictors of drug resistance

One potential mechanism that can confer pharmacological resistance is decreased drug exposure at the drug target, which can result from drug-drug interactions or inter-individual genetic variability (Figure 1.1A). There are a few well-established examples of germline genetics affecting exposure to anticancer therapies.[19] While outside the scope of this review, the importance of an established link between active drug exposure levels and clinical outcomes or adverse events must be emphasized. Drug exposure is predicted to affect drug efficacy or toxicity; however, discrete evidence must exist before clinical implementation is warranted.[40]

Somatic pharmacogenomics as a mechanism of drug resistance

Somatic mutations result in upregulation of oncogenic pathways, and their effects can be inhibited with the use of targeted therapies. Since 2003, over twenty PKIs have been approved to target various somatic alterations across a broad range of cancer types (including hematologic

and solid malignancies), and more than twenty additional PKIs are currently in clinical trials.[41] Because these drugs target protein kinases, somatic alterations in the targets or pathways may confer resistance or response (Table 1.1). Some well-established examples of somatic genetic drivers of resistance to PKIs are discussed below.

RAS status as a predictor of intrinsic resistance to EGFR inhibition in CRC

The epidermal growth factor receptor (EGFR or ErbB-1) is a transmembrane protein kinase that binds epidermal growth factor, inducing dimerization and autophosporylation, which signals downstream pathways (i.e., MAPK and PI3K/Akt) that mediate cellular proliferation and survival (Figure 1.2). The EGFR signaling pathway plays a pivotal role in tumor growth and progression in many cancer types, including glioblastoma, NSCLC, head and neck cancers, and CRC. EGFR is overexpressed in approximately 50% of CRC patients, and is associated with disease progression and poor prognosis.[42] Anti-EGFR monoclonal antibodies, such as cetuximab and panitumumab, were hypothesized to be effective in colorectal tumors over-expressing EGFR, and were initially U.S. Food and Drug Administration (FDA) approved for that indication; however, as monotherapy, the response rates to cetuximab and panitumumab were only 10% and 30%, respectively, indicating potential intrinsic resistance.[43,44] Retrospective analysis of phase III clinical study data revealed differential response to anti-EGFR monoclonal antibodies dependent on Kirsten rat sarcoma viral oncogene (*KRAS*) homolog.[45] When stratified by *KRAS* mutation status, response rate to panitumumab in wild-type individuals was 17%, whereas 0% of individuals with mutant *KRAS* responded. Similarly, *KRAS* mutations were associated with resistance and decreased overall survival (OS) in patients receiving cetuximab.[46] These observations provided evidence for *KRAS* as a biomarker of intrinsic resistance to EGFR-targeted monoclonal antibodies.

KRAS is a member of the rat sarcoma virus (*RAS*) family of oncogenes, which also includes *HRAS* (Harvey rat sarcoma viral oncogene homolog) and *NRAS* (neuroblastoma RAS viral oncogene homolog). Mutations in *RAS* genes lead to the constitutive activation of the MAPK pathway independent of *EGFR* status. Interestingly, in CRC, mutations in *KRAS* are significant enough to negate EGFR inhibition. A prospective-retrospective analysis of 1,183 patients who received either FOLFOX-panitumumab or FOLFOX alone revealed that mutation status in both *KRAS* and *NRAS* were predictive of response to panitumumab (HR for progression or death in *RAS* wild-type 0.72, 95% CI 0.58–0.99, p= 0.0004).[47] Recently, the American Society of Clinical Oncology released a provisional clinical opinion update recommending *NRAS* and *KRAS* mutation testing in CRC patients who are candidates for anti-EGFR monoclonal antibodies, supporting the significance of this biomarker as a predictor of intrinsic drug resistance.[48] Additional potential markers of intrinsic resistance have since been identified in models of *KRAS* wild-type patient xenografts, including *ERBB2* (*HER2*), *FGFR1*, *PDGFRA*, and *MAP2K1* (*MEK*); secondary mutations in *EGFR* at the site of cetuximab binding were identified in acquired resistance.[49] Further studies demonstrating effects on patient outcomes are needed before clinical implementation of these novel biomarkers can be recommended.

EGFR status as a mediator of resistance to EGFR PKIs in NSCLC

Mutations in *EGFR* are one of the most common cancer drivers identified in NSCLC tumors. Approximately 15% of NSCLC patients in the U.S. have an *EGFR* mutation, and the incidence is approximately 35% in patients of Asian descent.[50] In the U.S., *EGFR* mutations are most prevalent in females (17.9% vs. 8%, p= 0.002), non-smokers (42% vs. 6.6%, p < 0.001), and adenocarcinomas (15.6%).[51] The mutations are typically located in exons 18 to 21 of *EGFR*, the region that encodes the catalytic tyrosine kinase domain. Approximately 90% of the

mutations are short exon 19 deletions or the L858R point mutation in exon 21, which result in enhanced EGFR signaling.[52] Mutations in *EGFR* are predictive of response to EGFR inhibitors, such as erlotinib and gefitinib, in NSCLC. However, despite 70–80% response rates to EGFR PKIs, a majority of patients will acquire resistance after 10-12 months.[53,54] Absence of initial *EGFR* mutations confers lower overall response rates in NSCLC patients, a relative intrinsic resistance.[55,56]

Acquired resistance to EGFR inhibitors in NSCLC is complex and heterogeneous, but ultimately all mechanisms drive sustained signaling through downstream cancer pathways (e.g., MAPK or PI3K/Akt pathways). Known mechanisms of drug resistance include secondary genetic alterations in *EGFR*, upregulation of parallel signaling pathways, or phenotypic transformation (Figure 1.2). The most common mechanism of acquired resistance in EGFR-positive NSCLCs is the single-nucleotide mutation T790M, which occurs in approximately 50–60% of acquired resistance cases.[52] Also known as the gatekeeper residue, substitution of bulky methionine at this position causes steric hindrance and prevents EGFR inhibitors from binding and eliciting their pharmacologic effect. Other *EGFR* mutations have been identified in patients with acquired resistance, but their frequencies are much lower (Figure 1.3). Another common mechanism of acquired resistance to EGFR PKIs is amplification of the MET proto-oncogene (*MET*), a receptor tyrosine kinase, which has been reported in up to 22% of resistant samples.[57] *MET* amplification drives resistance by inducing EGFR-independent phosphorylation of ERBB3 (HER3), which activates downstream PI3K/Akt signaling despite the presence of an EGFR inhibitor.[57] Amplifications of *ERBB2* (*HER2*) and *PIK3CA* have also been identified in patients with acquired resistance, similarly functioning to activate shared pathways independent of EGFR.[14,58] Phenotypic transformation as a mechanism of acquired resistance occurs when the

histology of the tumor transitions to small cell lung cancer (SCLC) or from epithelial to mesenchymal subtype, and occurs in approximately 14% and 5%, respectively.[14,59] Interestingly, transformed SCLCs retain the primary *EGFR* activating mutations, but do not carry T790M or *MET* amplification. Not much is known about the mechanism of histological transformation, but currently it is recommended that these patients receive standard SCLC treatments.[60]

Numerous strategies are being investigated to overcome first-generation EGFR inhibitor resistance due to secondary *EGFR* mutations or bypass track signaling. One strategy was to develop more potent inhibitors of EGFR, such as the FDA-approved second-generation EGFR inhibitor, afatinib, which irreversibly binds to and inhibits EGFR as well as HER2, HER3, and HER4. Despite its first-line indication and increased potency, afatinib has not demonstrated promise in the setting of acquired drug resistance. LUX-Lung 1, a phase 2b/3 trial of afatinib in patients who had progressed after treatment with an EGFR PKI, failed to meet its primary endpoint of OS (HR 1.08, 95% CI 0.86-1·35; p=0.74), and response rates were less than 10%.[61] Demonstrating more promise than the second generation EGFR PKIs in combating acquired resistance are the third generation EGFR inhibitors: the recently approved osimertinib and rociletinib, which is in clinical trials. Like the second-generation inhibitors, these are irreversible EGFR inhibitors; however, they have higher affinity for mutant *EGFR*, including T790M, than for wild-type *EGFR*. In the pivotal phase 2 study of osimertinib in NSCLC patients who had progressed after treatment with a first generation PKI, response rates in T790M-positive patients were 61%. In a similar phase 1/2 study of rociletinib, patients with the T790M mutation had an objective response rate of 59%.[62,63]

Despite their therapeutic promise in acquired resistance to first generation EGFR PKIs, mechanisms of potential acquired resistance to third generation EGFR PKIs have already been

reported. A study in patients who progressed on rociletinib reported that half of T790M-positive NCSLCs treated with rociletinib became T790-wild-type at progression.[64] Loss of T790M was also observed in 27% patients who progressed on osimertinib, and 40% acquired a novel *EGFR* mutation, C797S, which is also resistant to rociletinib.[65] One possible strategy to overcome loss of T790M is combination therapy of an EGFR PKI with a monoclonal antibody that targets EGFR (e.g., NCT02496663). Recently, a preclinical study identified mutations and amplifications in *NRAS* and *KRAS* as mechanisms of acquired resistance to osimertinib.[66] Due to their upstream signaling of MEK, alterations in *NRAS* and *KRAS* may confer response to MEK inhibitors. As such, clinical studies investigating combination therapy with third generation EGFR PKIs and MEK inhibitors are also underway (e.g., NCT02580708).

Therapeutic strategies targeting resistance mediated by upregulation of bypass signaling pathways are less established. Postulated strategies mainly consist of studies investigating dual inhibition of EGFR and known bypass tracks. In an ongoing phase 1b/2 clinical study of gefitinib plus a novel MET inhibitor (INC280) in patients who progressed on a first-line EGFR PKI, partial responses were only observed in 6/41 (15%) individuals.[67] A phase 1b study investigated concurrent inhibition of HER2 and EGFR with the combination of afatinib (dual HER2/EGFR inhibitor) and cetuximab in NSCLC patients who had progressed on gefitinib or erlotinib, and overall response rate (ORR) was 29% and PFS was 4.7 months.[68] A phase 2 clinical trial combining erlotinib with a PI3K inhibitor (BKM120) is currently ongoing in patients who acquired resistance to erlotinib (NCT01487265); the combination of afatinib and sirolimus is also being studied to overcome resistance due to mTOR (mechanistic target of rapamycin, a serine/threonine kinase) activation (NCT00993499), a downstream component of the PI3K/Akt pathway. These preliminary results suggest that dual inhibition of multiple signaling pathways

may confer response in some individuals; however, an understanding of which individuals will benefit and durability of response is crucial.

Acquired resistance to ALK inhibitors

Anaplastic lymphoma kinase (*ALK*) is a receptor tyrosine kinase normally expressed in the nervous system that plays an important role in brain development. *ALK* rearrangements, mutations, and amplifications have been identified in numerous tumor types including anaplastic large cell lymphoma, neuroblastoma, and NSCLC. Chromosomal rearrangements resulting in gene fusions are the most common type of *ALK* alterations. Clinically, *ALK* alterations are known to be most actionable in NSCLC, where the most common rearrangement observed is echinoderm microtubule associated protein like 4 (*EML4)-ALK* .[69] Multiple variants of *EML4-ALK* have been reported; however, they all encode the same cytoplasmic portion of *ALK*, but have different *EML4* truncations.[70] Fusions of *ALK* with other genes have also been described, but occur at much lower frequencies.[71] These fusion proteins mediate ligand-independent dimerization of ALK, and like *EGFR* mutations, result in constitutive activation and downstream signaling through the MAPK and PI3K/Akt pathways.[69] Interestingly, *ALK* gene arrangements are largely mutually exclusive with *EGFR* or *KRAS* mutations.[72] Cancers harboring *ALK* rearrangements, classified as ALK positive, derive clinical benefit from ALK PKI therapies.

Crizotinib is a first generation ALK PKI, which also inhibits the MET and ROS proto-oncogenes (*MET* and *ROS1*), which encode receptor tyrosine kinases. Crizotinib first received accelerated FDA approval for ALK-positive NSCLC in 2011 based on durable, objective response rates of 61% in a single-arm phase 1 study, and is now recommended first-line in ALK-rearranged NSCLC.[73] Crizotinib inhibits ALK phosphorylation and signal transduction through G_1-S phase cell cycle arrest and induction of cellular apoptosis.[74] Despite high response rates

(74% in the first-line phase 3 study), resistance to crizotinib develops 10-12 months after therapy initiation.[75]

As observed with EGFR inhibitors, acquired resistance to crizotinib is heterogeneous and complex (Figure 1.3). Mechanisms of resistance that have been reported include somatic alterations (amplification and/or mutation) in *ALK*, activation of alternative signaling pathways, and genetic alterations in other important oncogenes. A case of phenotypic neuroendocrine transformation was recently reported in a patient who developed acquired resistance to crizotinib.[76] Unlike that observed in EGFR inhibitor resistance, only approximately one-third of patients with crizotinib resistance harbor an *ALK* mutation. Two of the most commonly observed mutations are L1196M and G1269. Amino acid 1196 is the gatekeeper residue of *ALK* and, similar to *EGFR*, it controls access to the active site; therefore, the bulky substitution of methionine causes steric hindrance, impeding crizotinib binding.[77] Individual mutations confer variable degrees of crizotinib resistance.[78,79] Activation of alternative signaling pathways, or bypass tracks, can occur via genomic or non-genomic mechanisms. These alterations lead to constitutive activation of redundant downstream pathways, rendering crizotinib incapable of suppressing tumor growth. Non-genomic mechanisms of resistance include increased phosphorylation of other tyrosine kinases (e.g., EGFR, IGF-1R, and Src), and genomic alterations include mutations in *EGFR*, *c-KIT*, *MAPK*, and *KRAS* (Figure 1.3).[80]

Multiple potential strategies exist to combat crizotinib resistance. The most well established second-line therapeutic option in patients who progress after receiving crizotinib is second-generation ALK inhibitors. Ceritinib, an FDA-approved second-generation ALK inhibitor, is 20-fold more potent against ALK than crizotinib and also inhibits IGF-1R (insulin-like growth factor 1 receptor). In a phase 1 clinical study, among the 80 patients who had

received crizotinib previously, there was a 56% response rate to ceritinib in patients with various *ALK* resistance mutations.[81] However, a preclinical study suggested that some crizotinib-resistance mutations (F1174C and G1202R) may not be sensitive to ceritinib and, in fact, may be mechanisms of acquired resistance to ceritinib as well.[82] Alectinib, another second-generation ALK inhibitor with efficacy in patients who progressed on crizotinib received FDA-approval December 2015.[83] Acquired mutations in *ALK* (I1171N and F1245C) confer resistance to alectinib, but are susceptible to ceritinib.[84,85] Interestingly, acquired resistance to lorlatinib (via *ALK* L1198F*)*, a third-generation ALK inihibitor in clinical trials, has been reported to enhance crizotinib binding and resensitize resistant tumors.[86] Findings such as these may be important when considering sequencing of therapy in resistant patients.

Strategies to overcome crizotinib resistance due to bypass track signaling are less understood; however, rational combination therapies have been postulated. For example, a preclinical study in ALK-positive NSCLC cell lines with acquired IGF-1R upregulation demonstrated improved efficacy of combined ALK (crizotinib) and IGF-1R inhibition (OSI-906).[87] In the case of EGFR-mediated mechanism of acquired resistance, combination therapy with an ALK and EGFR inhibitor has been suggested to be effective.[79,88] Other possible combinations include ALK inhibition concurrently with MEK or Src inhibitors. Heat shock protein 90 (HSP90) inhibitors have also demonstrated efficacy against ALK-sensitive and ALK-mutant NSCLCs.[79,89] HSP90 is a molecular chaperone that aids in proper folding of specific target proteins; ALK fusion proteins are substrates of HSP90. The combination of crizotinib plus HSP90 inhibitors is currently in clinical trials (NCT01712217).

Acquired resistance to BCR-ABL inhibitors in hematologic malignancies

Imatinib is an inhibitor of the *BCR-ABL* fusion gene, also referred to as the Philadelphia

chromosome, that is characteristic of CML and presents at lower frequencies in ALL and acute myeloid leukemia (AML). BCR-ABL is a constitutively active tyrosine kinase that functions by binding ATP and transferring a phosphate group to tyrosine residues to activate downstream signaling molecules. Imatinib competitively binds to the kinase pocket of BCR-ABL, inhibiting phosphorylation and downstream signaling.[90-92] The majority of CML patients will achieve clinically significant responses to imatinib. A complete and durable cytogenetic response is achieved in up to 80% of newly diagnosed patients and approximately 60% of patients with chronic-phase CML;[93,94] however, up to 27% of patients will develop resistance and relapse.[95]

Acquired resistance to imatinib occurs as a consequence of reactivation of BCR-ABL signaling, which can be due to *BCR-ABL* amplification, elimination of imatinib from the cell by multidrug efflux transporters, or, most commonly, by the development of *BCR-ABL* mutations.[96] The acquired mutations in *BCR-ABL* that have been associated with resistance to imatinib and other BCR-ABL inhibitors, such as nilotinib and dasatinib, are located in twelve key positions of *ABL1* (Abelson murine leukemia viral oncogene, homolog 1). These result in amino acid substitutions that change the BCR-ABL binding site, altering the ability of the PKI to bind and inhibit downstream signaling.[97] The most commonly observed *BCR-ABL* mutation conferring acquired resistance is T315I, followed by E255K/V. Interestingly, multiple acquired mutations in *BCR-ABL* have been identified, and the degree of imatinib (and other BCR-ABL PKI) resistance is dependent on the location of the point mutation within the BCR-ABL binding site.[98] Strategies to overcome acquired resistance to BCR-ABL PKIs are currently being explored.

The second-generation PKIs, such as nilotinib and dasatinib, were designed with a higher affinity for BCR-ABL in attempts to combat imatinib resistance. While they are able to overcome some mutations observed in acquired imatinib resistance, they are ineffective against

the common T315I mutation.[97] In 2012, a third generation BCR-ABL PKI, ponatinib, was approved. Ponatinib is unique in that it is a high affinity pan-BCR-ABL inhibitor with activity in T315I-positive CML. Despite its effectiveness against the acquired T315I mutation, emergence of compound mutations in *BCR-ABL* have been identified as conferring differential resistance to ponatinib.[97] Complex screening of resistance mutations and sensitivity to BCR-ABL PKIs is necessary to optimize therapy selection at the time of disease progression.

Acquired resistance to BCR-ABL inhibitors in solid tumors

Gastrointestinal stromal tumors (GISTs) are the most common mesenchymal tumors of the gastrointestinal tract, characterized by positive staining for *KIT* (the v-kit Hardy-Zuckerman 4 feline sarcoma viral oncogene). Approximately 95% of GISTs express *KIT*, 80% have mutations in *KIT*, and 10% have mutations in the platelet-derived growth factor receptor alpha (*PDGFRA*).[99] Wild-type GISTs lack *KIT* expression as well as *KIT* and *PDGFRA* mutations; alterations identified in wild-type patients include mutations in *BRAF*, *RAS*, *NF1*, and succinate dehydrogenase deficiency. Imatinib is first-line treatment in GIST due to its inhibition of KIT and PDGFRA. Furthermore, *KIT* and *PDGFRA* mutations are associated with response to imatinib, with 84% of mutation-positive patients achieving a partial response compared to 0% of wild-type patients.[100] Despite high initial response rates, approximately 80% of GIST patients develop imatinib resistance within 12–36 months.[99]

As observed in hematologic malignancies, acquired imatinib resistance in GIST is most commonly due to secondary alterations in the drug targets; however, because the oncogenic targets differ, so do the resistance alterations. A retrospective analysis of tumors from patients who progressed on the phase 2 imatinib trial indicated that 22 of 33 (67%) patients with acquired resistance had secondary mutations in *KIT* or *PDGFRA*.[101] Interestingly, secondary *KIT*

mutations were only observed in patients with primary *KIT* mutations, and the secondary *PDGFRA* mutation was identified in a patient with a primary *PDGFRA* mutation. The acquired mutations were all located within the ATP-binding pocket of KIT (e.g., T670I, the gatekeeper mutation) or the activation loop, thus inhibiting imatinib binding and inactivation of KIT-mediated signaling.

Current treatment strategies for acquired resistance to imatinib in GIST provide more potent inhibition of KIT and PDGFRA. Sunitinib, a multi-targeted PKI with activity against KIT, PDGFRA, VEGF, and numerous other tyrosine kinases, is currently approved second-line in patients with imatinib-resistant disease. Sunitinib has activity against all resistant genotypes, but response rates are significantly higher in wild-type GIST ($p= 0.04$) and patients with mutations in the ATP-binding domain ($p= 0.0005$) when compared to patients with alterations in the activation loop.[102] Efficacy of the third-line treatment, regorafenib, may be due to sufficient activity in patients with acquired alterations in the activation loop of KIT.[103] Despite their increased specificity for resistant disease, phase 3 clinical trial response rates to sunitinib and regorafenib were only 7% and 4.5%, and time to progression was 27 and 17 weeks, respectively.[104,105] These low response rates suggest that the majority of imatinib-resistant GISTs may be entirely resistant to KIT inhibition, require more potent inhibitors, or may require alternative inhibition strategies.

Mutations and amplifications of *KIT* are also observed in melanomas (~3%), with higher frequencies in mucosal (39%), acral (36%), and chronically sun-damaged (28%) subtypes, suggesting possible benefit from imatinib.[106] A phase 2 trial of imatinib in patients with *KIT*-positive metastatic melanoma demonstrated potential efficacy, with 53.5% of the patients achieving a response and a 6-month PFS rate of 36.6%.[107] Another phase 2 trial identified

differential response rates between melanoma patients with *KIT* mutations versus *KIT* amplification (54% vs. 0%, respectively).[108] Retrospective data from patients with *KIT*-mutated melanoma demonstrated potential efficacy of sunitinib; of 4 evaluable patients, 3 (75%) responded to sunitinib (1 complete remission and 2 partial responses).[109] Additional studies support use of sunitinib in mucosal and acral subtypes of melanoma, but failed to show an association between *KIT* status and response.[110,111] Despite clinical benefit and relatively high initial response rates, most patients eventually progress on these therapies.

Similar to hematologic malignancies and GIST, the current strategy to overcome acquired resistance to KIT inhibition in melanoma is the use of more potent inhibitors. Nilotinib, the BCR-ABL PKI used in chemoresistant CML, also has activity against KIT, PDGFR, DDR, and several other protein kinases, with greater potency than imatinib. Recently, a phase 2 study of niltonib demonstrated potential efficacy in patients with acquired resistance to prior KIT inhibitors. The primary endpoint, 4-month disease control, was achieved in 27% of resistant patients (95% CI 8% - 56%), and two partial responses (18.2%, CI 3% - 47%) were observed.[112] A similar phase 2 study of nilotinib in *KIT*-positive melanomas in a Korean population failed to meet its primary endpoint of response rate (overall response rate was 16.7%); however, six of the seven responses observed occurred in patients with *KIT* mutations only (24% response rate in *KIT*-mutated melanoma), suggesting that nilotinib may provide benefit in this subgroup.[113] As in GIST, *KIT* status seems to be a biomarker of response to imatinib in melanoma, but resistance inevitably develops. Current strategies to combat resistance are the same as GIST, with stronger inhibition of KIT, but response rates are low and not durable. Further understanding of the mechanisms of resistance and means to effectively suppress drivers of drug resistance are crucial to achieve durable responses in GIST and melanoma patients.

Acquired resistance to BRAF inhibition

BRAF, a serine/threonine kinase, plays a key role in the MAPK signaling pathway, which affects cell division, differentiation, and growth. Mutations in *BRAF* have been associated with numerous cancers, including CRC, melanoma, thyroid carcinoma, NSCLC, and non-Hodgkin's lymphoma. Most commonly observed are somatic mutations causing activation of BRAF, specifically a valine to glutamine or lysine substation at position 600 (V600E/K), and constitutive signaling through the MAPK pathway. The discovery of *BRAF* mutations in cancer led to development of drugs aimed at inhibiting this oncogenic driver. In 2011, the first selective BRAF inhibitor, vemurafenib, was FDA-approved for the first-line treatment of *BRAF V600E*-positive melanoma after interim review of a phase 3 randomized controlled trial that demonstrated improved six-month OS (84% vs. 64%) and PFS (5.3 months vs. 1.6 months).[114] Dabrafenib, a second BRAF inhibitor, demonstrated similar efficacy (PFS 5.1 vs. 2.7 months, HR 0.30, $p<0.0001$) and, was approved for the treatment of *BRAF*-positive melanoma in 2013; however, efficacy of BRAF inhibitors alone is not durable and most patients develop resistance within 6–8 months.[16,115]

Most mechanisms of acquired resistance to BRAF inhibitors involve reactivation of the MAPK pathway; unlike EGFR and ALK, no secondary or gatekeeper-type mutations have been identified as resistance drivers.[116] Secondary resistance may be driven upstream (e.g., upregulation and activation of the other receptor tyrosine kinases), downstream (e.g., activating *MEK1/2* mutations), at the level of *BRAF* (e.g., alternative splicing, *BRAF V600E* amplification), or elevated *CRAF* levels. Observed genetic alterations in the setting of acquired resistance include mutations that activate *NRAS*, *MEK1*, and *MEK2*.[116-118] Amplification of mitogen-activated protein kinase kinase kinase 8 (*MAP3K8* or *COT*) is another mechanism of resistance,

which results in RAF-independent activation of MEK and ERK signaling.[119]

One potential strategy to circumvent or delay BRAF inhibitor resistance is dual inhibition of components of the MAPK-pathway. In a phase 3 clinical trial of BRAF and MEK inhibition vs. BRAF inhibition alone in melanoma, the combination of dabrafenib plus trametinib improved PFS when compared to dabrafenib alone (9.3 vs. 8.8 months, HR 0.75, p= 0.03); significant improvements in response rates and OS were also observed.[120] Trametinib is now FDA approved as monotherapy or in combination with dabrafenib. Interestingly, the benefit of MEK inhibitors was not observed when administered as monotherapy in patients who had progressed after initial benefit from a BRAF inhibitor, suggesting that MEK inhibition alone is not sufficient to overcome BRAF resistance.[121] Preclinical data suggests another potential strategy to delay or overcome resistance caused by increased MAPK signaling is concurrent or sequential inhibition of BRAF and ERK; however, acquired resistance still occurs within 12 months.[122-124] Known mechanisms of acquired resistance to BRAF plus MEK inhibition are similar to those observed with BRAF inhibitor monotherapy, and include amplification of *BRAF V600*, which activates CRAF and subsequently MAPK signaling, and acquired *MEK1/2* mutations.[125-127] Preclinical studies investigating even broader combination therapy consisting of concurrent BRAF, MEK, and PI3K/mTOR inhibition have demonstrated potential efficacy in BRAF/MEK-induced resistance.[125,126,128] In addition, like *ALK*, *BRAF V600E* is a client of HSP90, and preclinical studies suggest that treatment with an HSP inhibitor may be another successful strategy to overcome BRAF and MEK inhibitor resistance.[129]

MAPK-independent mechanisms of acquired resistance to BRAF inhibition have also been identified, and dual inhibition has been proposed as a strategy to overcome this resistance. The most well studied bypass track of BRAF inhibitor resistance is activation through the

PI3K/Akt signaling pathway. For example, increased expression of *PDGFRB* or *IGF-1R* has been observed in cell culture and patient xenograft models of secondary resistance. Over-activation of these receptors results in activation of alternate signaling pathways (e.g., PI3K/Akt), which can reduce the cancer cells' dependency on MAPK signaling, rendering the cells resistant to BRAF-mediated inhibition.[116,130] Secondary mutations in PI3K pathway regulatory genes, such as *AKT1/3, PIK3CA, PIK3CG, PIK3R2*, and *PTEN* have also been observed, further supporting combination therapy with PI3K inhibitors.[131] Preclinical data demonstrated efficacy of concurrent PI3K and MEK inhibition in BRAF resistant cell lines, and clinical trials of this combination are currently underway (NCT01363232, NCT01337765). Recently, expression of yes-associated protein 1 (*YAP1*), a member of the Hippo signaling pathway, was associated with resistance to BRAF and MEK inhibition, and preclinical studies demonstrated that triple therapy with a BRAF, MEK, and YAP inhibitor may be a promising strategy to increase response in the setting of resistance.[132]

Mechanisms of resistance to multi-targeted PKIs

The multi-targeted PKIs, axitinib, cabozantinib, pazopanib, regorafenib, sorafenib, sunitinib, and vandetanib, represent a unique class of PKIs due to their inhibition of multiple diverse protein kinase targets.[133] This promiscuous inhibition results in broad efficacy across both solid and hematologic malignancies, but also contributes to numerous side effects that may be attributed inhibition of non-cancer driving pathways. While these agents demonstrated overall efficacy in clinical trials, approximately 20-30% of patients treated with multi-targeted PKIs experienced a best response of progressive disease, indicating no therapeutic benefit from the outset (intrinsic resistance).[104,134-140] Similarly, in a systematic review of twelve medical centers, Heng and colleagues observed that 26% of 1,056 renal cell carcinoma patients treated with

multi-targeted PKIs demonstrated intrinsic resistance.[141] Interestingly, evidence of cross-resistance to these agents has been demonstrated.[142] Despite their broad use in clinical practice, there is a paucity of data on mechanisms of resistance to the multi-targeted PKIs (Table 1.1). Using *in vivo* short hairpin RNA screening in mouse models of hepatocellular carcinoma (HCC), Rudalska and colleagues found that elevated Mapk14-Atf2 signaling predicted poor response to sorafenib.[143] Individual pharmacogenomic studies have also reported associations between germline mutations in drug transporters (*ABCG2*/BCRP and *ABCB1*/P-gp), drug metabolizing genes (*CYP3A5*), and pharmacodynamic genes (*VEGFR3*) with resistance; however, lack of replication to date limits clinical translation of these findings.[144-147] Lysosomal sequestration, resulting in insufficient active drug exposure, has been demonstrated as another potential mechanism of non-response to multi-targeted PKIs in multiple studies.[148,149] One study detected an association between increased interleukin 8, a pro-angiogenic cytokine, expression with acquired resistance to these agents.[150] Despite these individual findings, no biomarkers of intrinsic or acquired resistance to multi-targeted PKIs have demonstrated sufficient evidence to warrant implementation into practice. The overwhelming lack of studies using clinical data to explore somatic genetics as predictors of resistance to multi-targeted PKIs is also notable, especially considering that most of the biomarkers currently implemented within routine clinical practice for other agents fall within this category (Table 1.1).

Conclusions

Targeted therapies are increasingly common and recommended first-line in some cancer types due to their impressive increases in response rates and survival benefits when compared to standard cytotoxic chemotherapy. However, even in the presence of a genetic biomarker predictive of response, not all patients will benefit from such therapies (intrinsic resistance), and

for those who do, durable response rates are low (acquired resistance). Therefore, equally important as identifying targetable oncogenic alterations is the identification of biomarkers of intrinsic resistance and the ability to anticipate potential mechanisms of acquired resistance that may develop.

As discussed, the most common mechanisms of acquired resistance to targeted therapies induce upregulation of the drug target or bypass signaling through the same or similar pathways, resulting in cancer progression. These patterns imply an evolutionary-like process in which the cells most fit to regulate cellular proliferation and survival are selected. The question of whether or not resistant cells are present at undetectable concentrations at diagnosis or whether they develop post-treatment remains unanswered. Specifically, does resistance arise from pre-existing clones or is there drug-induced selection pressure that drives acquisition of mutations? It is likely that both may be the case.

An important concept in the context of resistance to anticancer therapies is that a biomarker conferring response or resistance in one tumor type may or may not be predictive in all tumor types. For example, approximately 10% of CRCs carry *BRAF V600* mutations, but are resistant to BRAF inhibition.[151,152] However, in a recent basket study of vemurafenib in *BRAF V600*-mutated non-melanoma cancers, which included seven tumor classifications, only patients with NSCLC and Erdheim-Chester disease or Langerhans'-cell histiocytosis met the predetermined overall response rate of >35%, suggesting that *BRAF* status may be important in those tumor types.[153] Recently, vemurafenib also demonstrated efficacy in *BRAF*-positive hairy cell leukemia.[154] Another example is *RAS*-status as a predictor of intrinsic resistance to EGFR monoclonal antibodies in CRC, but not predictive of response to EGFR inhibition in NSCLC. Interestingly, secondary mutations in *EGFR* have been reported as mechanisms of acquired

resistance in both tumor types. Lack of efficacy across tumor types may be reflective of differential oncogenic drivers or compensatory resistance mechanisms. In CRC, data suggests that BRAF inhibition is overcome through increased EGFR-mediated signaling; this bypass track is not clinically relevant in melanoma due to low basal levels of EGFR in this cancer type.[155] Therefore, dual BRAF and EGFR inhibition may be required to increase response rates in *BRAF*-mutant CRC.[156,157] The benefit of imatinib in hematologic malignancies with *BCR-ABL* translocations and solid tumors with *KIT* or *PDGFRA* mutations is a unique example of how different oncogenic drivers may respond to the same therapy, but result in different genetic mechanisms of acquired resistance.

Future directions

Treatment modalities to prevent and overcome drug resistance are critical to increase the rate of durable responses to cancer therapies (Figure 1.4). To date, most strategies used in clinical practice involve sequential dosing once resistance develops (Figure 1.4A). An alternative strategy is to predict and target known resistance pathways from the outset using combination therapy (Figure 1.4B). The strength of this approach is supported by the increased benefit of dabrafenib plus trametinib vs. dabrafenib alone in *BRAF*-mutated melanoma.[158] However, while this prolongs duration of response, resistance inevitably develops. Studies indicate development of BRAF inhibitor dependence in melanoma cells, which may also be combatted with intermittent or continuous dosing (Figure 1.4C).[159,160] Another possible strategy is pulse dosing, in which the targeted therapy is administered for a short time (maybe until progression), stopped temporarily, and then restarted (Figure 1.4D). This method is supported by serial biopsies of *EGFR*-positive NSCLC resistance which demonstrated that patients may re-develop the T790M mutation after withdrawal and, therefore, respond to re-initiation of an EGFR inhibitor.[14] Finally,

some patients with advanced disease may benefit from immunotherapy, but identification of this subset is much less understood.

Despite overall response rates of 50% to 80% in clinical trials of targeted anticancer therapies, there is a paucity of data on mechanisms of intrinsic resistance. Intrinsic resistance may be due to germline genetics (e.g., *BIM* or *TPMT*) or somatic alterations (e.g., *KRAS* in CRC). Drug metabolism and germline variants that may affect exposure to cancer therapies are also an important consideration in the context of intrinsic resistance.[19,161] However, robust examples of intrinsic resistance to targeted anti-cancer therapies are lacking. Furthermore, mechanisms of resistance to PKIs that inhibit multiple targets, such as sorafenib and sunitinib, are even more challenging to elucidate due to the heterogeneity of their effects, and are even less studied (Table 1.1). Another interesting gap in the area of resistance to anticancer therapies involves potential racial disparities. Certain alterations are more common in some races (e.g., *EFGR* mutations in Asian populations), suggesting potential differences in racial trends of oncogenic drivers. Because clinical and genetic studies enroll a vast majority of Caucasian patients, these potential disparities in genetic oncogenesis between races, which can greatly affect response rates, have yet to be elucidated.

Tables and Figures

Table 1.1: Summary of FDA-approved protein kinase inhibitors and known mechanisms of resistance

Class	Drug	Generation	Indication	Indication subgroup	Innate (primary) resistance	Acquired (secondary) resistance
EGFR inhibitors	erlotinib gefetinib afatinib	1st 1st 2nd	NSCLC NSCLC NSCLC	EGFR exon 19 deletions or exon 21 (L858R) substitution mutations	EGFR wildtype, BIM deletion	secondary EGFR mutations (e.g., T790M), MET amplification, HER2 amplification (*see Fig. 3*)
	osimertinib	3rd	NSCLC	EGFR T790M-positive	EGFR wildtype	loss of T790M, secondary EGFR mutations (e.g., C797S)
	cetuximab panitumumab	monocolonal antibodies	CRC head and neck	KRAS wildtype	RAS mutations	secondary mutations in EGFR, RAS or BRAF; activation of HER2 or MET
ALK inhibitors	crizotinib	1st	NSCLC	ALK+, recommended for pts with ROS1 translocation	ALK wildtype	secondary ALK mutations, ALK fusion amplification, bypass signaling (e.g., EGFR, KIT, IGF1R) (*see Fig. 3*)
	ceritinib	2nd	NSCLC	ALK+, resistant to crizotinib, ALK I1171N+	ALK wildtype	secondary ALK mutations (e.g., G1202R)
	alectinib	2nd	NSCLC	ALK+, resistant to crizotinib		secondary ALK mutations (e.g., I1171N)

BCR-ABL inhibitors	imatinib	1st	CML, ALL, MDS, GIST	Ph+	BIM deletion; KIT and PDGFRA wildtype (GIST)	BCR-ABL1 T315I and others in heme; KIT and PDGFR secondary mutations in GIST
	bosutinib dastinib nilotinib	2nd 2nd 2nd	CML CML, ALL CML	Ph+	BCR-ABL1 T315I	If used first-line, BCR-ABL1 T315I and others
	ponatinib	3rd	CML, ALL	Ph+ for whom no other PKI is indicated, or **T315I positive**		BCR-ABL1 compound mutations
BTK inhibitor	ibrutinib		CLL, MCL, WM		C481S, point mutations in phospholipase Cy2 (PLCg2)	Mutations in BTK and downstream
HER2 inhibitor	lapatinib		breast	HER2+		deregulation of PIK3CA pathway, AXL over-expression
BRAF inhibitors	dabrafenib		melanoma	BRAF V600E or V600K	RAC1 mutations, loss of PTEN or NF1, CCND1 over-expression, abundance of HGF	re-activation of the MAPK pathway, activating mutations in NRAS, activating MEK 1/2 mutations, elevated CRAF (*see Fig. 3*)
	vemurafenib		melanoma	BRAF V600E		
MEK inhibitor	trametinib		melanoma	BRAF V600E or V600K		

Multi-targeted VEGF inhibitors	axitinib cabozantinib pazopanib regorafenib sorafenib sunitinib vandetanib	RCC thyroid thyroid RCC, sarcoma CRC, GIST HCC, RCC, thyroid thyroid			
mTOR inhibitor	everolimus	breast, pNET, RCC, angiolyolipoma, SEGA	breast HER2-	TSC1/TSC2 mutations predict *response*	
ER inhibitors	tamoxifen, fulvustrant				ESR1 mutations
HER2 inhibitor	trastuzumab	breast, gastric	HER2+	PIK3CA mutations possibly predictive of response in neoadjuvant setting (not predictive in adjuvant)	
SMO inhibitor	vismodegib	basal cell carcinoma			SMO mutations

Figure 1.1: Mechanisms of oncology drug resistance. (A) Pharmacological resistance. Drug-drug interactions and germline pharmacogenetic variants can affect drug exposure at the tumor site. Pharmacological properties can affect drug penetration into the central nervous system. (B) Biological resistance. Somatic (acquired) mutations in the drug target can affect the drug's ability to effectively inhibit oncogenesis. Somatic alterations downstream of the drug target can result in constitutive upregulation of oncogenic pathways. Genetic alterations may also activate alternative oncogenic signaling pathways. Some tumor types may transform into other tumor types (e.g., non-small cell lung cancer to small cell lung cancer). (C) Pharmacological drug resistance results from inadequate drug levels at the site of action, whereas biological drug resistance results despite adequate drug levels at the site of action. (D) Intrinsic resistance is the lack of even transitory clinical benefit – the tumor continues to progress despite treatment. (E) Acquired resistance is the lack of tumor response to medication despite initial benefit.

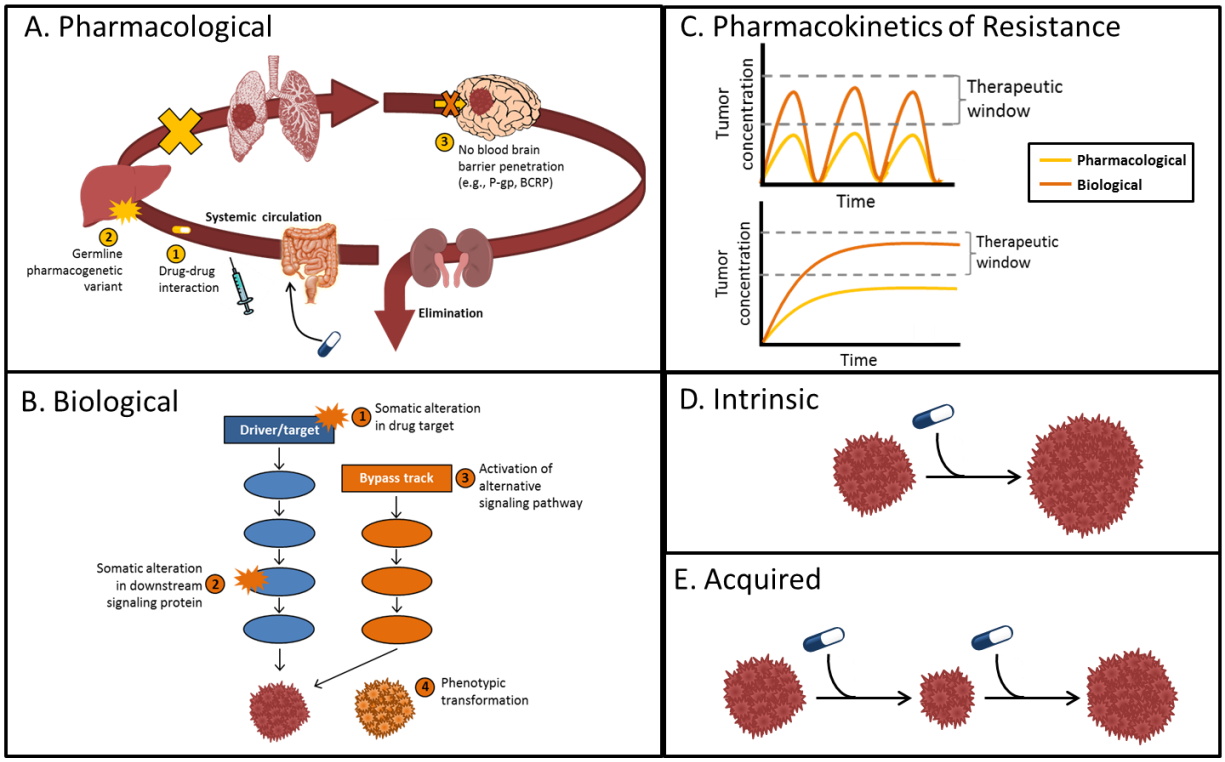


Figure 1.2: Example mechanisms of resistance to EGFR inhibitors and potential treatment strategies: The MAPK and PI3K/AKT pathways. EGFR-mutated non-small cell lung cancers show initial response to EGFR inhibitors (e.g., erlotinib, gefetinib). Mechanisms of acquired resistance include secondary EGFR mutations, downstream mutations that result in EGFR-independent activation of MAPK or PI3K/AKT signaling pathways, or mutations in alternative protein kinases that bypass EGFR-mediated signaling through MAPK and/or PI3K/AKT pathways. Potential treatment strategies to combat acquired resistance to EGFR inhibitors include stronger inhibiton of EGFR (e.g., afatinib), combination therapy with a MEK (e.g., trametinib) or mTOR (e.g., sirolimus) inhibitor, or inhibition of bypass tracks.

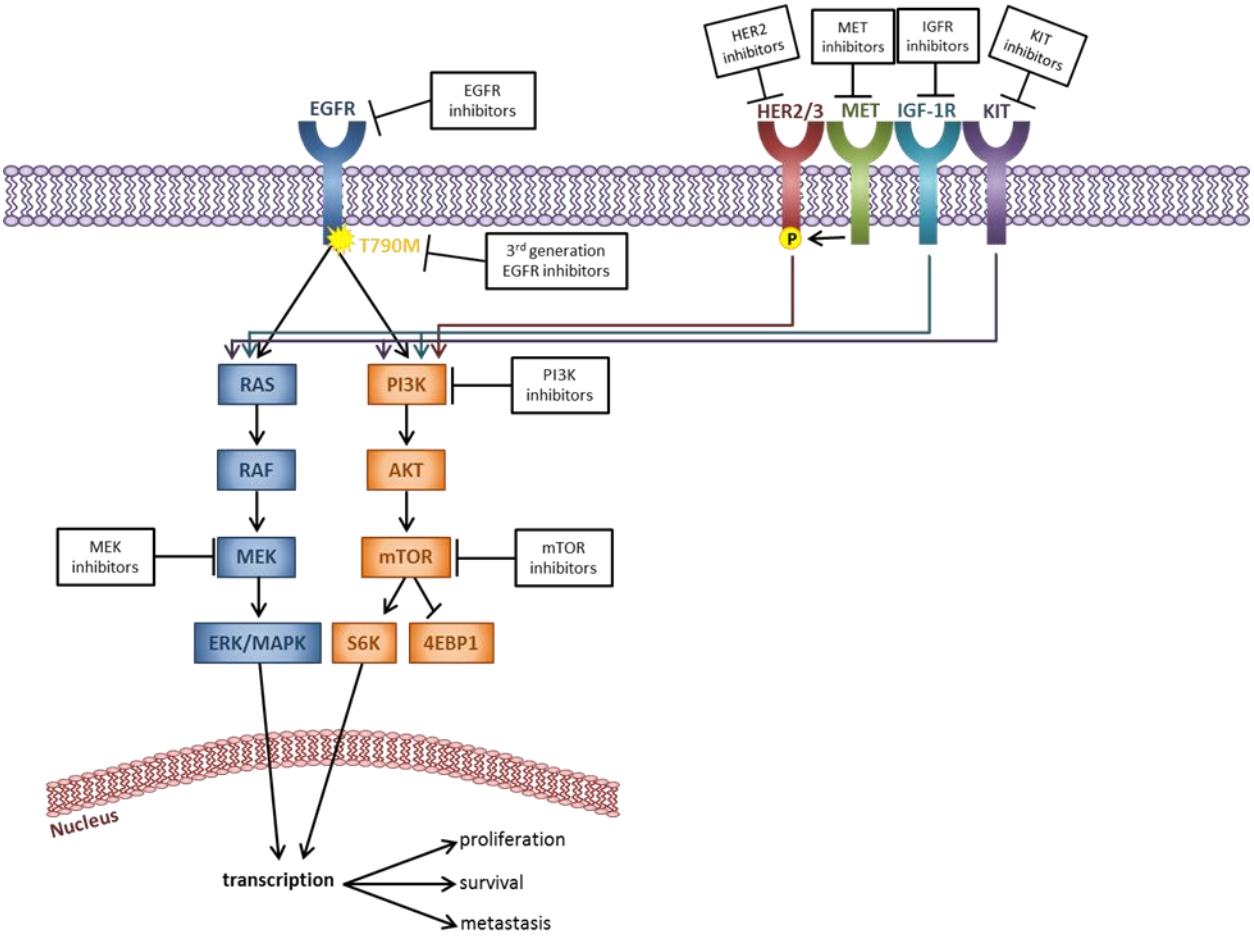


Figure 1.3: Mechanisms of acquired resistance to protein kinase inhibitors. (A) and (B) data from Camdige et al., 2014. (C) Data from Shi et al., 2013. The all-blue pie chart represents MAPK-mediated mechanisms of resistance. PI3K/AKT-mediated mechanisms include AKT1/3 mutations (3%), mutations in positive-regulatory genes, PIK3CA and PIK3CG, and mutations in negative-regulatory genes PIK3R2, PTEN, and PHLPP1.

Blue shades throughout correspond to alterations within the targeted oncogenic track; orange shades correspond to alterations regulating alternative or bypass tracks; yellow shades correspond to phenotypic transformations. NSCLC: non-small cell lung cancer, SCLC: small cell lung cancer, EMT: epithelial to mesenchymal subtype.

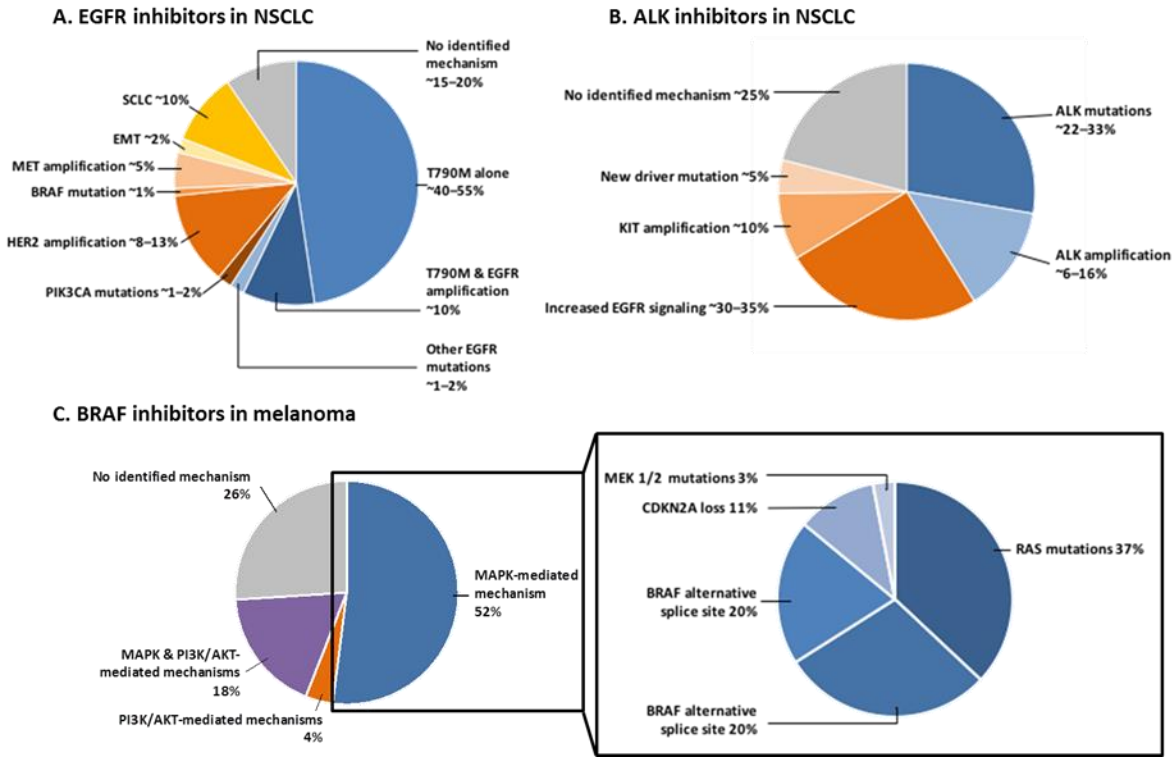
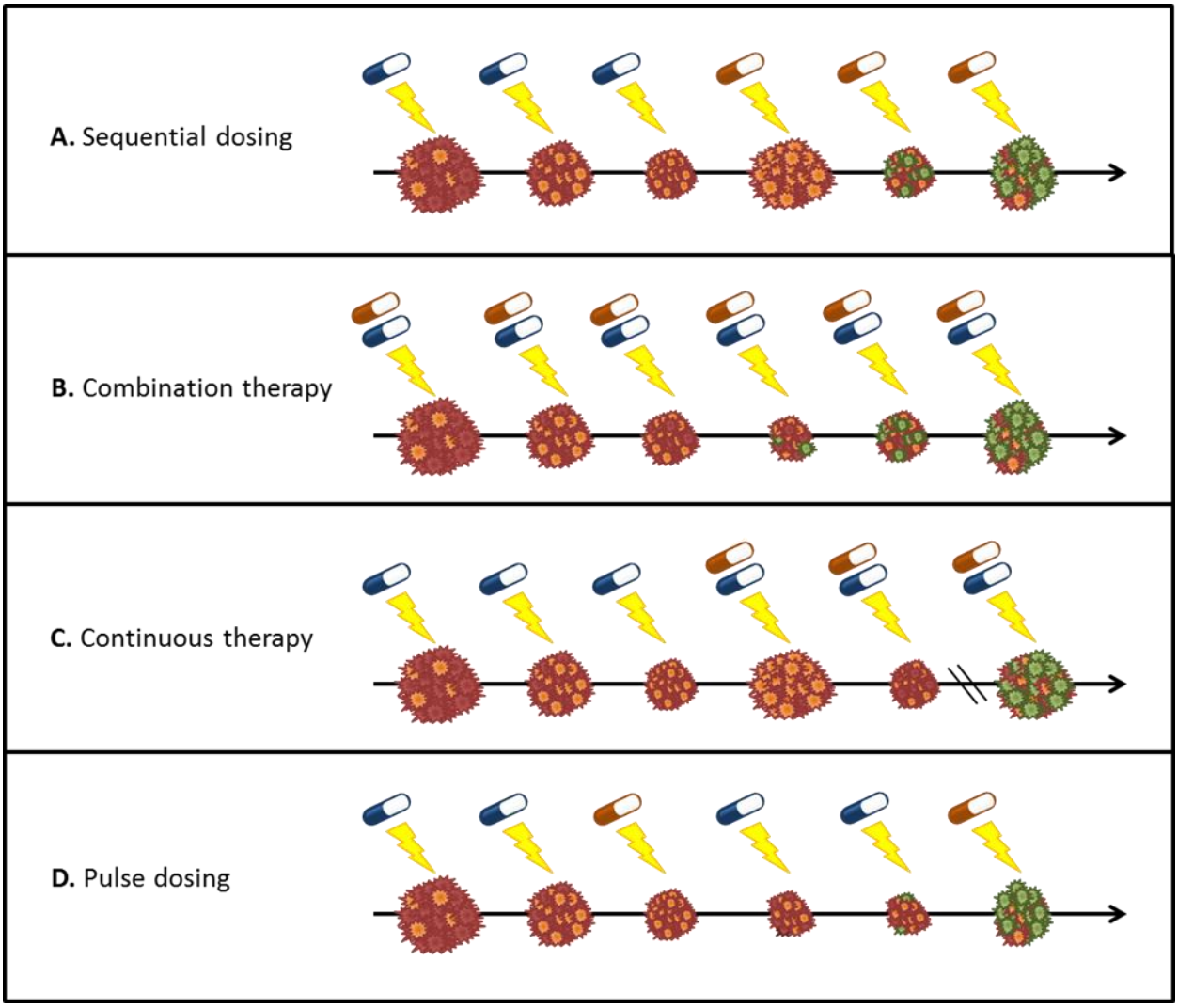


Figure 1.4: Potential treatment strategies to combat anticancer drug resistance. (A) Sequential dosing. Begin treatment with single targeted therapy, once resistance develops, switch to drug that targets resistant cells. (B) Combination therapy. Begin therapy with a medication that targets the identified oncogenic marker in combination with a drug targeting predicted resistance mechanisms. (C) Continuous therapy. Begin treatment with a single targeted therapy, once resistance develops, add medication that targets resistance mechanism. (D) Pulse dosing. Begin treatment with single targeted therapy and periodically administer medication that targets predicted resistance mechanism. (A) represents the most commonly used strategy to date. Some data suggest that (B), (C), and/or (D) may delay development of acquired resistance.



SPECIFIC AIMS

AIM 1: Understand demographic and clinical factors that may influence activity of multi-targeted tyrosine kinase inhibitor (TKI) therapy.

1a: Perform a large-scale retrospective study to explore the proposed association between multi-targeted TKI administration and the life-threatening adverse events, Stevens-Johnson syndrome and toxic epidermal necrolysis. This may elucidate potential risks of multi-targeted TKI treatment that contribute to lack of therapeutic benefit. *Discussed in Chapter 2*

1b: Perform a retrospective cohort study to explore the potential association between demographic and clinical characteristics with intrinsic resistance to multi-targeted TKIs. This will provide information on whether certain subsets of patients are less likely to derive benefit from multi-targeted TKI therapy. *Discussed in Chapter 3*

AIM 2: Understand the impact of somatic genetics on resistance to multi-targeted TKIs.

2a: Conduct a methodology evaluation study to assess the utility of a diverse collection of formalin-fixed paraffin-embedded samples as a source for somatic genetic information. Execution of this aim will inform sample selection for genetic analysis sub-aims. *Discussed in Chapter 4*

2b: Utilize next-generation sequencing to identify somatic point mutations that are associated with intrinsic resistance to multi-targeted TKIs. This will be the first analysis aimed at elucidating genetic mechanisms of intrinsic resistance to the widely prescribed multi-targeted TKIs. *Discussed in Chapter 5*

2c: Detect somatic copy number variations that are associated with intrinsic resistance. This will provide insight into another potential genetic mechanism of intrinsic resistance in the context of multi-targeted TKIs. *Discussed in Chapter 5*

AIM 3: Develop an initial genetic model to pre-emptively identify patients resistant to multi-targeted TKIs. Completion of this aim will provide a preliminary decision algorithm that, with validation, can be easily translated into clinical practice to pre-emptively distinguish patients unlikely to respond to multi-targeted TKIs, thus guiding clinical decision-making toward other therapeutic options. *Discussed in Chapter 5*

CHAPTER 2: EXPLORING THE PROPOSED LINK BETWEEN TYROSINE KINASE INHIBITORS AND THE INCIDENCE OF STEVENS-JOHNSON SYNDROME AND TOXIC EPIDERMAL NECROLYSIS IN A LARGE CANCER PATIENT COHORT[2]

Summary

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are rare life-threatening mucocutaneous reactions most commonly triggered by medications. Case reports and small case series have suggested that cancer patients may be at increased risk of experiencing SJS/TEN, but a comprehensive assessment had not been performed. Additionally, multiple published case reports linked tyrosine kinase inhibitors (TKIs) to SJS/TEN reactions, and multi-targeted TKIs have been reported in over thirty individuals in the FDA Adverse Event Reporting System. We conducted a retrospective cohort study to determine whether cancer patients are at increased risk for SJS/TEN and to explore the potential association of TKI administration with SJS/TEN. Cancer patients (n = 108,825; 2002-2015) were screened for ICD-9 codes suggestive of SJS/TEN. Chart reviews were conducted to confirm diagnoses, and culprit drugs for cases were abstracted from medical records. A total of 121 patients were identified with potential SJS/TEN reactions. Diagnosis was confirmed in 20 individuals, possible diagnoses in 30 individuals, and 12 individuals reported historical diagnoses, corresponding to an average annual incidence of 6-15 cases per 100,000 individuals. The most common trigger was antibiotics, followed by antineoplastics and anticonvulsants. Three patients were treated with TKIs closely

[2] This article is updated from an article submitted for publication. The citation for the article under review is as follows: Gillis NK, Hicks JK, Bell GC, Kanetsky PA, McLeod HL. Incidence and triggers of Stevens-Johnson syndrome and toxic epidermal necrolysis in a large cancer patient cohort. 2017. *In review*.

preceding or at the time of SJS/TEN reaction; one possible SJS/TEN case was linked to administration of a multi-targeted TKI. This corresponds to a prevalence of 20-60 cases per 100,000 individuals in patients treated with TKIs, which may indicate a heightened risk with TKIs; however, inherent challenges to confirm SJS/TEN diagnosis and culprits warrant additional investigation. Our data demonstrate that cancer patients may be at up to a 150-fold increased risk for SJS/TEN compared to the general population.

Introduction

Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) are life-threatening mucocutaneous skin reactions most commonly attributed to medications. They are variants of erythema multiforme, characterized by extensive necrosis and detachment of the epidermis, and are classified by percentage of body surface involved (SJS involves less than 10%, SJS/TEN overlap involves 10-30%, and TEN involves greater than 30%). SJS and TEN are extremely rare, with incidence in the general population ranging from 1-2 cases per million per year, although increased risk is associated with certain genetic alterations, diseases, and medications.[162-164] Due to the life-threatening nature of SJS/TEN, early identification and withdrawal of the causative agent(s) is crucial to maximize prognosis. Of the over two hundred culprit drugs identified, the medications most commonly known to trigger SJS and TEN include xanthine oxidase inhibitors, aromatic anticonvulsants, nonsteroidal anti-inflammatory drugs, and sulfonamide antibiotics.[165] Non-pharmacological risk factors reported include inherited genetics (i.e., *HLA* status), vaccinations, infections, and HIV/AIDS.[165-167]

Case reports and small case series have suggested that cancer patients may also be at increased risk of experiencing SJS/TEN, but a comprehensive assessment has not been

performed. A recent large observational study on the epidemiology of SJS/TEN in a medically diverse population suggested that active cancer may be one factor associated with increased risk.[168] Another review of 32 TEN cases found that cancer was the most common comorbidity (9 patients, 28%).[169] A systematic literature review identified 83 published reports of anticancer drugs implicated as culprits for SJS/TEN reactions.[170] While the study found only a statistically significant association of SJS with bendamustine, multiple other anticancer medications were linked to cases of SJS or TEN, including immunomodulators (e.g., thalidomide, lenolidamide, methotrexate), tyrosine kinase inhibitors (TKIs), and cytotoxic antineoplastics (e.g., docetaxel). Multiple case reports have also linked TKIs, including the multi-targeted TKIs, to SJS/TEN reactions.[171-180] As of February 2017, the multi-targeted TKIs have been reported in thirty-seven cases in the FDA Adverse Event Reporting System, with sorafenib having the most associations (17 cases of SJS or TEN).

We conducted a retrospective study to identify the incidence and triggers of SJS/TEN in a large cancer patient cohort. We compared the incidence of SJS/TEN in our cancer patient cohort to that reported in the general population. We conducted detailed medical chart reviews to explore cases for possible links with TKIs.

Methods

Patients were identified through a retrospective search of electronic health records at Moffitt Cancer Center. Moffitt Cancer Center's Institutional Review Board approved this study and waived the requirement for informed consent. All cancer patients treated at Moffitt Cancer Center from June 15, 2002 through June 15, 2015 and who had International Classification of Disease (ICD-9) codes available in the electronic health records were eligible for study inclusion. The ICD-9 codes 695.1 (erythema multiforme, which encompassed SJS/TEN pre-2009), 695.12

(erythema multiforme major), 695.13 (SJS post-2009), 695.14 (SJS/TEN overlap post-2009), and 695.15 (TEN post-2009) were utilized to identify patients who may have experienced SJS/TEN.

Retrospective chart reviews of identified patients were completed independently by two clinicians to confirm SJS/TEN diagnosis. Patients' allergies, oncologist clinical notes, medication lists, dermatology consult notes (when applicable), and pathology notes (when applicable) were reviewed to confirm SJS/TEN diagnoses and identify any culprit drug(s). Patients were classified independently according to chart reviews as (1) confirmed SJS and/or TEN diagnosed at Moffitt Cancer Center, (2) possible SJS and/or TEN [included cases diagnosed at an outside hospital while a patient at Moffitt and uncertain differential diagnoses (e.g., SJS vs. drug eruption)], (3) historical (occurring prior to coming to Moffitt) SJS and/or TEN, or (4) failure to confirm SJS and/or TEN diagnosis. When necessary, adjudication of conflicting conclusions was completed through collaborative chart reviews and discussion. Culprit drugs were abstracted from chart reviews and represent the hypothesized triggers according to the patients' physicians. Concomitant TKIs at the time of SJS/TEN were also recorded.

The primary outcome was average annual incidence of SJS/TEN over the 13-year period. Annual incidences were calculated as the number of unique patients with a SJS/TEN diagnosis divided by the total number of patients screened that year. Incidences were calculated for confirmed SJS/TEN cases (condition 1) and confirmed or possible SJS/TEN cases (conditions 1 and 2). Timing of historical cases (condition 3) relative to cancer diagnoses was unknown, so these cases were excluded from calculations. Prevalence of SJS/TEN in patients receiving TKIs was calculated as number of patients on TKIs at time of SJS/TEN diagnosis divided by the total number of patients screened who had been prescribed a TKI. Demographic (e.g., age, sex, and race) and clinical factors (e.g., cancer type and hematopoietic stem cell transplantation status)

were collected to determine incidence rates within groups.

Results

Incidence of SJS and TEN

A total of 108,825 unique cancer patients were screened for SJS/TEN ICD-9 codes in the electronic health records. ICD-9 codes corresponding to potential SJS and/or TEN were identified in 121 patients. Due to electronic billing system implementation in 2003, the number of patients screened in 2002 (the denominator for incidence) is uncertain, so 2002 cases are excluded from the incidence calculations. Manual chart review confirmed SJS/TEN diagnosis at Moffitt Cancer Center (condition 1) in 20 patients, possible SJS/TEN (condition 2) in 30 patients, and historical SJS/TEN diagnoses (condition 3) in 12 patients (Table 2.1). SJS and/or TEN was ruled out or unconfirmed (condition 4) in 59 patients. The average annual incidence of confirmed cases of SJS/TEN (condition 1) at Moffitt Cancer Center was 5.9 (range 0-15.6) cases per 100,000 individuals; the average annual incidence of confirmed and possible cases SJS/TEN (conditions 1 + 2) was 15.3 (range 0-28.3) cases per 100,000 individuals (Table 2.2). Confirmed cases of SJS/TEN were more common in hematologic diagnoses (i.e., leukemias, lymphomas, multiple myeloma, and MDS) compared to solid malignancies [0.09% (14/15,813) vs. 0.007% (6/90,567), respectively]. Interestingly, 5 of the 20 (25%) confirmed cases occurred in patients with acute myeloid leukemia. Of the confirmed cases of SJS/TEN, 35% (7/20) had undergone hematopoietic stem cell transplantation prior to SJS/TEN diagnosis, 57% (4/7) of which were allogeneic.

Triggers of SJS and TEN

Classifying agents according to drug class, there were 69 physician-reported culprits for the 62 confirmed, possible, and historical cases of SJS/TEN; the trigger for 6 patients was

unknown. Physician-reported triggers for SJS/TEN diagnoses included antibiotics, anticonvulsants, anticancer agents, anti-gout, antifungals, and immunomodulators, amongst others (Figure 2.1). The most common trigger in all identified cases of SJS/TEN was antibiotics (33/62 cases, 53.2%), with trimethoprim/sulfamethoxazole being the most frequently stated culprit (14/62 cases, 22.6%). Anticonvulsants and antineoplastic agents were the second most commonly stated trigger (6/62 cases, 9.7% each). Antineoplastics attributed to SJS/TEN reactions included temozolomide, hyper-CVAD combination therapy, and irinotecan, amongst others, which were each attributed to one case. Immunomodulatory antineoplastics included interferon (n = 1), methotrexate (n = 2), and lenalidomide (n = 1). The miscellaneous agents, each attributed to one historical case of SJS/TEN, were bisphosphonates, estrogen or ibuprofen, epoetin alfa, saline, and clobetasol. (See Appendix 2.1 for a detailed list of all attributed triggers.)

Of the 121 SJS/TEN diagnoses codes, 10 (8.3%) individuals received treatment with a TKI; however, 70% of the cases definitively could not be linked to the SJS/TEN reaction (4/10 initiated the TKI after SJS/TEN diagnosis and in another 3/10, SJS/TEN diagnosis was ruled out). Of the three remaining cases that could potentially be linked to TKI use, only one possible case (condition 2) was attributed to a TKI by the physician; the patient was blinded to treatment on a clinical trial of sorafenib vs. sunitinib, both of which are multi-targeted TKIs, was diagnosed with erythema multiforme of unspecified degree, and treatment was discontinued. For the remaining two cases, one was treated with erlotinib one month prior to SJS/TEN, which was attributed to penicillin, and the other was treated with dastinib three months prior to SJS/TEN, which was attributed to trimethoprim/sulfamethoxazole. This corresponds to possible SJS/TEN prevalence of 20-60 cases per 100,000 individuals (1-3 cases/4,977 patients prescribed a TKI) in

individuals treated with TKIs.

Discussion

We utilized our institutional electronic health system biorepository of over 100,000 patients to determine the incidence and identify potential triggers of SJS/TEN in cancer patients. In our study cohort, the average annual incidence of SJS/TEN was 6-15 cases per 100,000 per year. Most conservatively, this corresponds to a 25-60-fold higher incidence in cancer patients than reported in the general population; leniently, the incidence observed is 77-150-fold higher in cancer patients. Other diseases, such as HIV, also have a heightened incidence of SJS/TEN compared to the general population.[181] An elevated incidence of SJS/TEN has also been observed in specific geographic regions, in particular East Asia, likely due to the presence of high susceptibility HLA loci.[182] Overall, reported triggers of SJS/TEN in our cancer patient cohort were similar to those known to be associated with the reaction in the general population, and included antibiotics, anticonvulsants, and anti-gout agents; however, some cases of SJS/TEN were attributed to cancer-specific therapies, including antineoplastics, monoclonal antibodies, and immunomodulators. We observed a relatively low prevalence of SJS/TEN in patients receiving TKIs, with only one possible case of SJS/TEN being linked to TKI use. Due to the relatively small number of patients prescribed a TKI in our cohort, we have insufficient evidence to draw a definitive conclusion regarding the possible association between TKI use and SJS/TEN. While we cannot confirm a heightened risk of SJS/TEN, challenges to culprit drug attribution exist. Physicians are likely to preferentially attribute SJS/TEN reactions to drugs with reported associations, and, due to the severity of the reaction, patients will never be re-challenged with potential triggers, so culprits cannot be confirmed and causality is not definitive. If all three cases of individuals on TKIs were, in fact, associated with TKI usage, this would

correspond to an approximately 600-fold increased risk compared to the general population.

Cancer patients have been shown to have increased mortality rates from SJS/TEN when compared to individuals without cancer,[183] therefore, a thorough understanding of the factors that increase risk of SJS/TEN in cancer patients is especially critical to facilitate early culprit withdrawal and maximize patient outcomes. The immunocompromised state of many cancer patients, the observation of immunomodulators as a trigger, and the higher incidence of SJS/TEN in HIV patients suggest a possible role of the immune system in the increased risk of SJS/TEN.[167] Other possible explanations include increased exposure to culprit medications, and the cancer disease process, or synergy between risk factors.

A notable strength of our study was the utilization of electronic medical records to confirm ICD-9 code diagnoses. It has been demonstrated that use of claims databases to investigate incidences of SJS results in significant overestimates; in fact, Strom and colleagues found that only 14.8% of individuals with SJS claims had a final diagnosis of SJS.[184] Likewise, in our study we found that only 16.5% (20/121) of individuals with SJS/TEN ICD-9 codes had confirmed diagnoses. The use of claims data alone would have resulted in a 600 to 1200-fold higher incidence in cancer patients being reported.

Limitations of our study are inherent to the challenges of treating SJS. First, there are no definitive diagnostic criteria for SJS/TEN, so it is possible that cases of SJS/TEN were missed during our screening phase that relied on querying ICD codes. Additionally, as stated above, culprits cannot be confirmed and causality cannot be definitively determined. Variable follow-up times may have contributed to a decreased observation of cases, further contributing to the conservative nature of the estimate reported. Future studies are warranted to determine the role of the cancer disease process, immune system, and culprit drugs on SJS/TEN pathogenesis in

cancer patients. Ultimately, a comprehensive SJS/TEN prediction algorithm, incorporating drugs, immune system status, and relevant genetics (e.g., HLA type), may assist in identifying high-risk patients and facilitating early detection and withdrawal of causative agents to maximize patient outcomes.

Tables and Figures

Table 2.1: Patient demographics for individuals screened for Stevens-Johnson syndrome. Summary information is provided for entire cohort screened and all possible cases of Stevens-Johnson syndrome (SJS) and/or toxic epidermal necrolysis (TEN), sorted by classification based on manual chart review. Incidence is presented for confirmed cases (condition 1) and possible cases (condition 2).

Characteristic	Patients screened* (n = 108,825)	Confirmed cases (n = 20)	Possible cases (n = 30)	Historical cases (n = 12)	All cases (n = 62)
Age[#]	-	53 (29-78)	58 (23-76)	60 (32-80)	59 (23-80)
Sex					
Female	53,789 (49%)	7 (35%)	18 (60%)	6 (50%)	31 (50%)
Male	55,033 (51%)	13 (65%)	12 (40%)	6 (50%)	31 (50%)
Unknown	3 (<0.01%)	0	0	0	0
Race					
White	97,846 (90%)	18 (90%)	27 (90%)	12 (100%)	57 (92%)
Black	5,547 (5%)	1 (5%)	2 (7%)	0	3 (5%)
Asian	1,279 (1%)	1 (5%)	0	0	1 (2%)
Other	4,153 (4%)	0	1 (3%)	0	1 (2%)
Ethnicity					
Non-Hispanic	97,423 (89.5%)	17 (85%)	25 (83%)	12 (100%)	54 (87%)
Hispanic	7,088 (6.5%)	2 (10%)	4 (13%)	0	6 (10%)
Unknown	4,314 (4%)	1 (5%)	1 (3%)	0	2 (3%)
Cancer Type					
Solid	90,567 (83%)	6 (30%)	18 (60%)	9 (75%)	33 (53%)
Hematologic	15,813 (14.5%)	14 (70%)	10 (33%)	3 (25%)	27 (44%)
Unknown	2,445 (2%)	0	2 (7%)	0	2 (3%)
Prevalence of SJS/TEN	NA	18/100,000	28/100,000	11/100,000	57/100,000

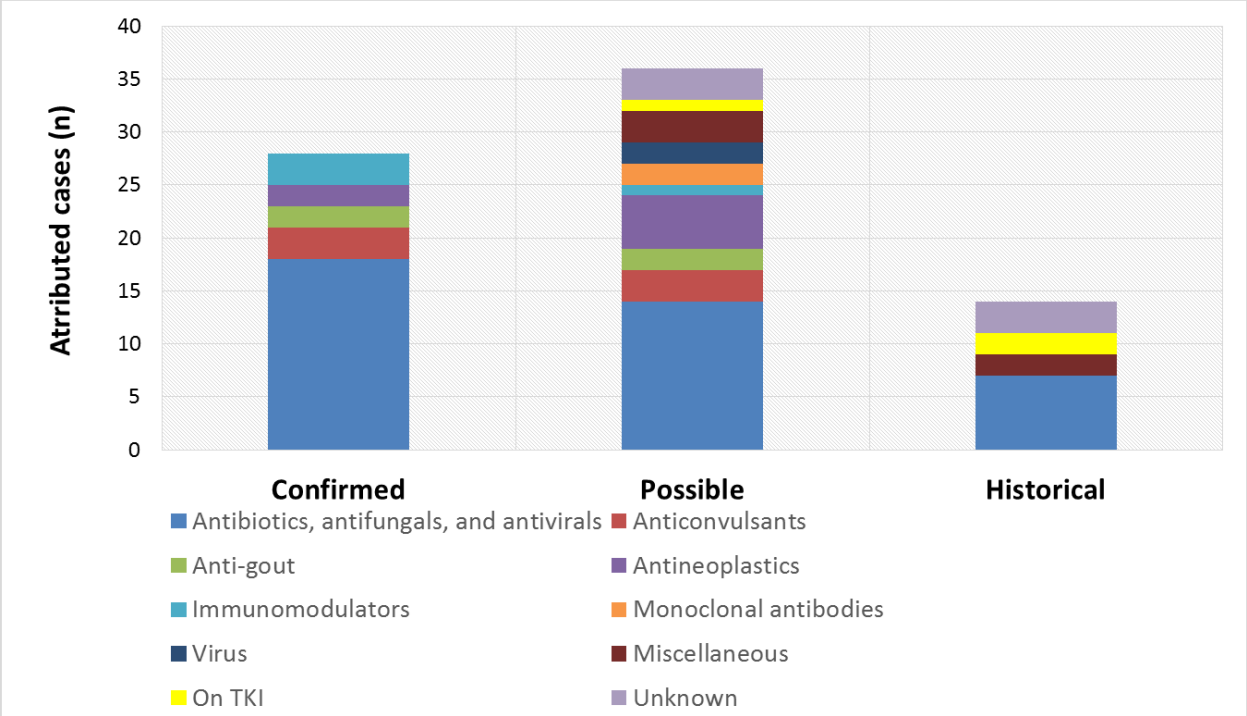
*Patients were initially screened under a de-identified protocol, so age at diagnosis is unavailable. Cancer type represents primary site as reported in Florida Cancer Registry.
[#]Age listed represents the median and range at time of SJS/TEN ICD-9 code

Table 2.2: Incidence of Stevens-Johnson syndrome and toxic epidermal necrolysis by year.

	Minimum			Maximum		
Time period	Confirmed only	# Screened	Incidence/ 100,000	Confirmed + Possible	# Screened	Incidence / 100,000
6/15 - 12/31/02	2	*	*	4	*	*
2003	0	14,125	0	4	14,125	28.32
2004	3	19,193	15.63	3	19,193	15.63
2005	3	21,365	14.04	4	21,365	18.72
2006	1	25,411	3.94	5	25,411	19.68
2007	0	26,499	0	3	26,499	11.32
2008	0	23,683	0	0	23,683	0
2009	2	25,244	7.92	4	25,244	15.85
2010	1	18,763	5.33	3	18,763	15.99
2011	1	28,313	3.53	5	28,313	17.66
2012	1	31,124	3.21	3	31,124	9.64
2013	2	32,116	6.23	4	32,116	12.45
2014	3	33,001	9.09	6	33,001	18.18
1/1 - 6/15/15	1	25,092	3.99	2	25,092	7.97
01/01/03-6/15/15	18	323,929[#]	5.56	46	323,929[#]	14.2
Average annual incidence			**5.91**			**15.3**

*Electronic billing system implementation in 2003 complicates denominator determination for 2002.
#Due to patients being treated over multiple years, this sum does not represent unique patients screened, rather it is the total number of patients screened during all periods.

Figure 2.1: Triggers of Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN). Triggers were identified from patient medical records and are reported according to SJS/TEN occurrence: confirmed cases (n = 20 patients), possible cases (n = 30) and historical cases (n = 12). For some patients, there was not a single definitive trigger, resulting in attribution to and discontinuation of multiple medications, which results in a higher number of attributed cases on the y-axis than number of cases in some groups (confirmed: 28, possible: 35, and historical: 12). Three patients (yellow) were on TKIs closely preceding or at time of SJS/TEN diagnosis.



CHAPTER 3: IDENTIFYING POTENTIAL DEMOGRAPHIC AND CLINICAL FACTORS PREDICTIVE OF RESISTANCE TO MULTI-TARGETED TYROSINE KINASE INHIBITORS

Summary

Multi-targeted tyrosine kinase inhibitors (TKIs) are widely prescribed antineoplastic agents that provide significant benefit in overall and progression-free survival across a wide range of cancer types. Despite their overall efficacy, approximately 20% to 30% of individuals with clinical indication for a multi-targeted TKI will not respond, demonstrating intrinsic resistance. We conducted a retrospective cohort study to identify potential demographic and clinical features associated with intrinsic resistance to multi-targeted TKIs. Chart reviews of 266 cancer patients treated with multi-targeted TKIs were completed and clinical imaging studies, radiologists', and oncologists' impressions of disease before and after TKI initiation were evaluated to classify patients as resistant or non-resistant. Demographics, including age, sex, race, and ethnicity, and clinical factors, including tumor type, TKI received, and duration of treatment, were compared between resistant and non-resistant patients. We observed an overall resistance rate of 21% in our cohort of patients receiving multi-targeted TKIs in the context of routine clinical practice. There were no significant differences in the demographics or clinical features of resistant versus non-resistant patients, other than duration of multi-targeted TKI treatment, which was significantly longer in non-resistant compared to resistant patients (11 mo vs. 3.6 mo, FDR p-value = 1.1×10^{-6}). In conclusion, the phenomenon of intrinsic resistance to multi-targeted TKIs observed in both clinical trials and routine practice was not explained by differences in demographics, tumor type, or TKI received in our cohort. Similar rates of

resistance in the subgroups analyzed suggest that there may be other unidentified shared markers of resistance to these agents.

Introduction

Tyrosine kinase inhibitors (TKIs) are widely prescribed antineoplastic agents that provide significant benefit in progression-free and overall survival in a wide range of solid tumors and hematologic malignancies. A subset of TKIs, the multi-targeted TKIs function by inhibiting multiple tyrosine kinases known to be important in regulation of oncogenic cell proliferation, differentiation, and survival (Table 3.1). The promiscuity of these agents results in broad efficacy across cancer types, as well as potential for adverse effects driven by inhibition of off-target, non-cancer driving tyrosine kinases. Therefore, a benefit-to-risk assessment is crucial to inform personalized decision-making when prescribing these medications.

The clinical trials that led to approval of the multi-targeted TKIs demonstrated overall improvements in progression free and overall survival, however, a significant percentage of individuals in each clinical trial did not derive even transient benefit from these agents – demonstrating intrinsic resistance. For example, in the phase III clinical trial that led to approval of sorafenib for the treatment of advanced hepatocellular carcinoma, 21% of individuals in the sorafenib treatment arm experienced progressive disease from the outset.[136] Similarly, in the phase III GRID trial for regorafenib in advanced gastrointestinal stromal tumors (GIST), 20% of individuals discontinued treatment at first follow-up due to disease progression.[104] The phase III clinical trial for cabozantinib in medullary thyroid cancer demonstrated similar resistance rates with 26% of individuals in the active treatment arm discontinuing therapy due to progressive disease.[185]

Pre-emptive identification of individuals who may respond to multi-targeted TKIs versus those not likely to benefit, but only be subjected to adverse effects, is critical to optimize the benefit-to-risk assessment when prescribing these medications. The aforementioned clinical trials performed analyses to identify potential demographic and clinical factors (e.g., sex, race, ethnicity, tumor type, etc.) associated with response to active drug versus placebo, however, analyses have not been performed within the active drug arms to identify potential prognostic factors characteristic of individuals who did not respond to active treatment. The primary objective of this study was to identify potential demographic and clinical factors that may be predictive of intrinsic resistance to multi-targeted TKIs. We conducted a retrospective cohort study of individuals treated with multi-targeted TKIs within routine clinical practice and explored differences in demographic and clinical features of individuals who demonstrated intrinsic resistance compared to those who achieved any degree of response (i.e., stable disease, partial response, or complete response).

Methods

Patient population

Patients were identified from the Total Cancer Care (TCC) cohort at Moffitt Cancer Center, an institutional review board (IRB)-approved biobanking protocol in which individuals agree to provide tissue and blood samples for research and to be followed throughout their lifetime.[186] Additional IRB approval was granted to conduct this particular study in the TCC cohort (MCC #18603). Individuals eligible for study inclusion were patients consented between January 1, 1994 and December 31, 2015 over the age of 18 years at diagnosis, who had a solid tumor diagnosis and were treated at Moffitt Cancer Center with a multi-targeted TKI (axitinib, cabozantinib, pazopanib, regorafenib, sorafenib, sunitinib, or vandetanib). Exclusion criteria

included individuals treated with a single-targeted TKI (e.g., gefitinib, which selectively targets the epridermal growth factor receptor or crizotinib, which selectively targets the anaplastic lymphoma kinase, etc.) and individuals treated with a multi-targeted TKI for a hematologic malignancy, due to challenges in determining response in the adjuvant setting.

Data collection

Data was initially abstracted in a standardized manner from the TCC biorepository system (TransMed, Cupertino, CA). Data abstracted from TransMed included demographic variables (date of birth, sex, race, and ethnicity) as well as clinical information, including date of diagnosis, primary site at diagnosis, histology, first course of treatment, multi-targeted TKI(s) received, and start and stop date of TKI(s). Sources for these data were Florida Cancer Registry and electronic medical records (Appendix 3.1). Information abstracted from TransMed was used to guide manual chart reviews.

Chart reviews were conducted by two independent clinicians and researchers to verify abstracted data and generate treatment phenotypes for eligible individuals. Patients were classified as either "resistant" or "non-resistant" based on clinical response to first multi-targeted TKI received. Specifically, the date of initiation of the first multi-targeted TKI received was collected from clinical notes and recorded; the tumor type being treated with the first multi-targeted TKI was also noted. Imaging scans (including PET, CT, MRI, and ultrasounds) conducted most recently prior and soonest after TKI initiation were reviewed. Radiologists' and oncologists' assessments of patient response were utilized to determine whether each patient derived some clinical benefit from the TKI (non-resistant) or progressed despite TKI treatment (intrinsically resistant). If a patient had imaging at any point after TKI initiation that demonstrated clinical response, they were classified as non-resistant; a trial period of at least one

month was required to determine resistance to TKI treatment. Independently derived phenotypes (i.e., resistant vs. non-resistant) were compared between reviewers. In the case of discrepancies, adjudication was conducted by a third clinician and researcher (Jeffrey Russell, MD, PhD).

Data analysis

This was an exploratory study which included 266 patient cases identified in the TCC biorepository. Non-parametric statistics were employed as to not assume normality of our data. Patient demographics between intrinsically resistant and non-resistant individuals were compared using contingency tables and Fisher's exact test for categorical variables, and Mann Whitney U test for continuous variables. Odds ratios and 95% confidence interval (CI) were calculated using logistic regression. A false-discovery rate (FDR) correction was used to correct for multiple comparisons, and an FDR-corrected p-value less than 0.05 was considered significant. A post-hoc power calculation was conducted using an FDR-corrected threshold of $\alpha = 0.00714$, $\beta = 0.8$, and incorporating variable proportions of individuals with exposures to account for differences in variables (Table 3.2). Statistical analyses were conducted using R (version 3.2). A box and whisker plot and histogram were created in GraphPad Prism 6, and a forest plot was generated using SAS (version 9.3).

Results

Patient population

The approval timeline of multi-targeted TKIs (Table 3.1) resulted in a recruitment period of 2005 – 2016. A total of 266 patient charts were reviewed for this study. Of those charts reviewed, four patients first received a multi-targeted TKI to treat a hematologic malignancy and were excluded from further analyses. The demographics and clinical characteristics of the study cohort were as expected based on the patient demographics at Moffitt Cancer Center and the

FDA-approved indications and off-label uses of multi-targeted TKIs (Table 3.3). The median age of the study cohort was 56 years old (interquartile range 47-66). There were slightly more males (170/262, 65%) than females (92/262, 35%), and the vast majority of the population self-indicated as white (247/262, 94%) non-Hispanic (242/262, 92%). The primary tumor types being treated with the multi-targeted TKIs were renal cell carcinoma (RCC, 137/262 or 52%) and sarcoma, not otherwise specified (including GIST, 53/262 or 20%). Thirty-two patients (12%) were prescribed, but never started, a multi-targeted TKI, so were excluded from resistant versus non-resistant classification.

Clinical factors and associations with resistance

Of the 230 evaluable patients, 45 (20%) could not be classified as resistant or non-resistant because drug discontinuation was attributed to side effects only and no imaging studies were available to assess response (n = 21), patient was lost to follow-up (n=15), patient was not on therapy long enough to assess response (n = 6), or, specifically, patient died before response was assessed (n = 3). Of the remaining 185 evaluable patients, 39 (21%) were classified as resistant and 146 (79%) were classified as non-resistant.

There were no statistically significant differences in the age, sex, race, or ethnicity of resistant and non-resistant patients (Table 3.4). Neither tumor type being treated nor multi-targeted TKI received were associated with resistance (FDR-corrected p-value = 0.59 and 0.49, respectively). As an exploratory observation, tumor types that demonstrated higher rates of resistance than would be expected based on published literature included hepatocellular carcinoma (2/6 or 33% resistance), melanoma (3/7 or 43% resistance), and pancreatic neuroendocrine (4/11 or 36% resistance); patients treated with pazopanib also demonstrated higher resistance rates than would be expected (6/17 or 35%) (Figure 3.1). Odds ratios

demonstrated essentially no difference in resistance by age (OR = 0.98, 95% CI 0.96 – 1.01) or race (OR = 0.93, 95% CI 0.093 – 4.96) (Figure 3.2). Females were slightly more likely to be non-resistant than males (OR = 0.54) and non-Hispanics were slightly more likely to be resistant than Hispanics (OR = 1.37), however, these findings did not reach statistical significance (95% CI = 0.21 – 1.29 and 0.36 – 7.79, respectively). Duration of multi-targeted TKI administration was significantly longer in non-resistant patients compared to resistant patients (11 mo vs. 3.6 mo, FDR p-value = 1.1×10^{-6}, Figure 3.3).

Discussion

We conducted an exploratory retrospective study to identify demographic and clinical features associated with intrinsic resistance to multi-targeted TKIs. Our overall observations of this routine clinical practice cohort closely mimicked that observed in large phase III clinical trials. We observed an intrinsic resistance rate of 21% in our study cohort, and approximately 20% of individuals discontinued treatment early due to intolerable side effects.[104,136,185] Demographic factors explored for association with resistance included age, sex, race, and ethnicity; clinical factors of interest were tumor type/indication and multi-targeted TKI received. We did not identify any statistically significant associations between the demographics or clinical factors with intrinsic resistance. However, there was a highly significant association between duration of multi-targeted TKI administration and resistance, with resistant patients discontinuing therapy 35% sooner than non-resistant patients. Significance of this finding supports our phenotyping methodology and reiterates the dichotomy of responses to multi-targeted TKIs. Furthermore, the time on therapy for resistant patients (median = 3.6 mo) represents valuable time that patients were put at risk for adverse effects while not receiving therapeutic benefit.

This exploratory study suggests that intrinsic resistance to multi-targeted TKIs cannot be predicted based on demographics, cancer type/indication, or multi-targeted TKI administered. This finding is echoed by the results of the phase III clinical studies, which, when stratifying by demographic and clinical features, found that the multi-targeted TKIs provided significantly better outcomes than placebo or standard of care across all subgroups.[104,134,138,139,185] For example, Cox proportional hazards analysis of progression-free survival in pancreatic neuroendocrine patients receiving sunitinib versus placebo resulted in hazard ratios (HR) of 0.37 (95% CI 0.2 – 0.7) for males and 0.48 (95% CI 0.2 – 0.9) for females, 0.49 (95% CI 0.3 – 0.9) for whites and 0.35 (95% CI 0.2 – 0.7) for non-whites.[138] The similarity in the effect sizes and variability of the parameters provides support for the lack of association of these variables with response or resistance. Some of these clinical studies also performed molecular analysis in attempts to identify molecular predictors of response. The study of cabozantinib in thyroid carcinoma explored *RET* mutational status as a predictor of response and found that response rates were similar in *RET* mutated and *RET* wildtype individuals (objective response rates = 32% and 25%, respectively).[185] Similarly, the GRID study found no differences in response in individuals harboring common *KIT* mutations (HR 0.21 and 0.24).[104]

Despite clinical relevance, there is a paucity of data exploring resistance to multi-targeted TKIs. To date, there have been no successful studies identifying predictive markers of response or resistance to these widely prescribed medications. In this study we conducted an exploratory analysis in attempts to identify demographic and clinical factors that may be predictive of resistance. While failure to detect a significant association with any of the factors explored and resistance may be due to insufficient statistical power, based on similar observations in clinical studies, we believe that these characteristics are not informative in predicting response to these

agents. Additionally, the finding that there were no significant differences in resistance rates based on demographic characteristics, tumor type, or TKI received, suggests that there may be other *shared* factors that contribute to resistance to these agents. Additional studies are warranted to identify robust predictive markers of resistance to multi-targeted TKIs that can be used to optimize personalized treatment recommendations and maximize patient outcomes.

Tables and Figures

Table 3.1: FDA-approved multi-targeted tyrosine kinase inhibitors. Information provided includes FDA-approved and off-label indication(s), approval date(s), and select drug target(s). Abbreviations – CRC: colorectal carcinoma, GIST: gastrointestinal stromal tumor, HCC: hepatocellular carcinoma, pNET: pancreatic neuroendocrine tumor, RCC: renal cell carcinoma

Drug	FDA-approved indications (approval date, mo/yr)	Off-label uses	Select drug targets
axitinib	RCC (1/2012)	thyroid cancer	KIT, PDGFR, VEGFR1-2
cabozantinib	RCC (4/2016) thyroid cancer (1/2012)		KIT, FLT3, MET, RET, VEGFR1-3
pazopanib	RCC (10/2009) soft tissue sarcoma (4/2012)	thyroid cancer	KIT, PDGFR, VEGFR1-3
regorafenib	GIST (2/2013) CRC (2/2013)	HCC	BRAF, KIT, PDGFR, RET, TEK, TIE2, VEGFR1-3
sorafenib	HCC (11/2007) RCC (12/2005) thyroid cancer (3/2013)	angiosarcoma GIST	BRAF, CRAF, FLT3, KIT, PDGFR, VEGFR1-2
sunitinib	GIST (1/2006) pNET (5/2011) RCC (1/2006)	thyroid cancer soft tissue sarcoma	CSF1, FLT3, KIT, PDGFR, RET, VEGFR1-3
vandetanib	thyroid cancer (4/2011)		EGFR, FGFR, RET, VEGFR1-2

Table 3.2: Post-hoc power calculation. Alpha represents an FDR-corrected alpha level. The p0 represents the proportion of individuals with the exposure. For example, with a 10% proportion of females, an odds ratio (OR) of approximately 5 could be detected with these defined statistical thresholds.

Cases (n)	Controls (n)	alpha	beta	p0	OR
39	146	0.00714	0.8	0.1	5.053
39	146	0.00714	0.8	0.2	4.047
39	146	0.00714	0.8	0.3	3.853
39	146	0.00714	0.8	0.4	4.001
39	146	0.00714	0.8	0.5	4.525
39	146	0.00714	0.8	0.6	5.906
39	146	0.00714	0.8	0.7	11.808

Table 3.3: Patient demographics.

Characteristic	Number (%), n = 262
Age at diagnosis	56 (47-66)
Sex	
Male	170 (65)
Female	92 (35)
Race	
White	247 (94)
Black	10 (4)
Asian	4 (2)
Unknown	1 (0)
Ethnicity	
Hispanic	20 (8)
Non-Hispanic	242 (92)
Diagnosis (tumor type)	
Brain	6 (2)
Breast	8 (3)
Colorectal	4 (2)
Gynecologic	5 (2)
Hepatocellular	9 (3)
Melanoma	11 (4)
Pancreatic neuroendocrine	14 (5)
Renal cell	137 (52)
Sarcoma	53 (20)
Thyroid	6 (2)
Other	9 (3)
TKI received	
axitinib	4 (2)
cabozantinib	1 (0)
pazopanib	20 (8)
sorafenib	74 (28)
sunitinib	131 (50)
N/A	32 (12)

Continuous data is presented as median (interquartile range).

Table 3.4: Comparison of patient demographics and clinical characteristics by phenotype.

Characteristic	Resistant, N (%) (n=39)	Non-resistant, N (%) (n=146)	P-value	FDR-corrected P-value
Age at diagnosis	59 (47-68)	55 (46-64)	0.22*	0.49
Sex			0.18	0.49
Male	30 (77)	94 (64)		
Female	9 (23)	52 (36)		
Race			1.0	1.0
White	37 (95)	138 (95)		
Other	2 (5)	8 (5)		
Black	*1 (50)*	*7 (88)*		
Asian	*1 (50)*	*0*		
Unknown	*0*	*1 (13)*		
Ethnicity			0.77	0.90
Hispanic	3 (8)	15 (10)		
Non-Hispanic	36 (92)	131 (90)		
Diagnosis (tumor type)			0.42	0.59
Brain	1 (3)	3 (2)		
Breast	0	4 (3)		
Colorectal	0	3 (2)		
Gynecologic	1 (3)	4 (3)		
Hepatocellular	2 (5)	4 (3)		
Melanoma	3 (8)	4 (3)		
Pancreatic neuroendocrine	4 (10)	7 (5)		
Renal cell	16 (41)	80 (55)		
Sarcoma	11 (28)	29 (20)		
Thyroid	0	5 (3)		
Other	1 (3)	3 (2)		
TKI received			0.28	0.49
axitinib	0	4 (3)		
pazopanib	6 (15)	11 (8)		
sorafenib	10 (26)	51 (35)		
sunitinib	23 (59)	80 (55)		
Duration of TKI treatment (mo)	3.6 (2-5.3)	11 (6.8-18.1)	<0.0001*	1.1×10^{-6}

Continuous data is presented as median (interquartile range).
*****P-value calculated using Mann Whitney U test.

Figure 3.1: Observed resistance rates by clinical characteristics. Gray dashed lines represent the commonly reported range of non-responders in clinical trials. No bar corresponds to zero resistant individuals in our cohort. Sample sizes for each classification are presented in Table 5. Abbreviation – pNET: pancreatic neuroendocrine tumor.

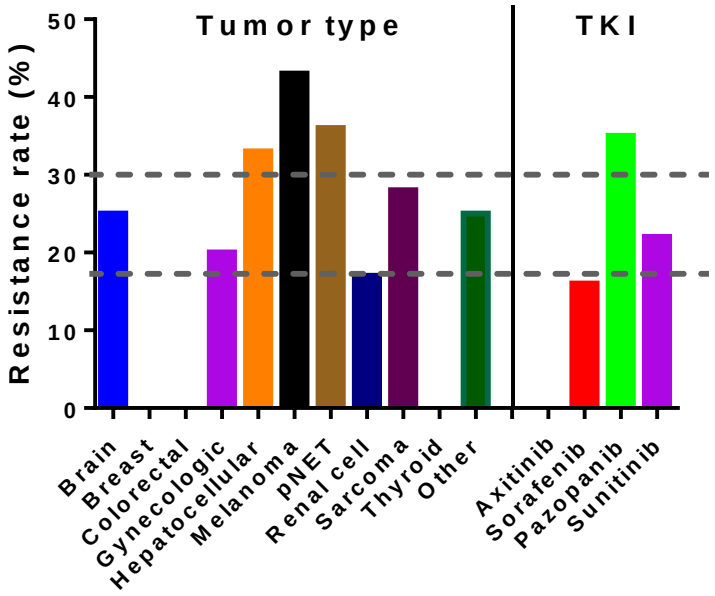


Figure 3.2: Forest plot of the relationship between demographic features and resistance. Blue diamonds represent odds ratios (OR) and bars represent the upper and lower 95% confidence intervals (95% CI). An OR greater than one favors the resistance phenotype, while an OR less than one favors the non-resistance phenotype. Reference groups for sex, race, and ethnicity were female, non-white, and non-Hispanic, respectively. Duration corresponds to the amount of time, in months, that individuals remained on the first multi-targeted tyrosine kinase inhibitor prescribed.

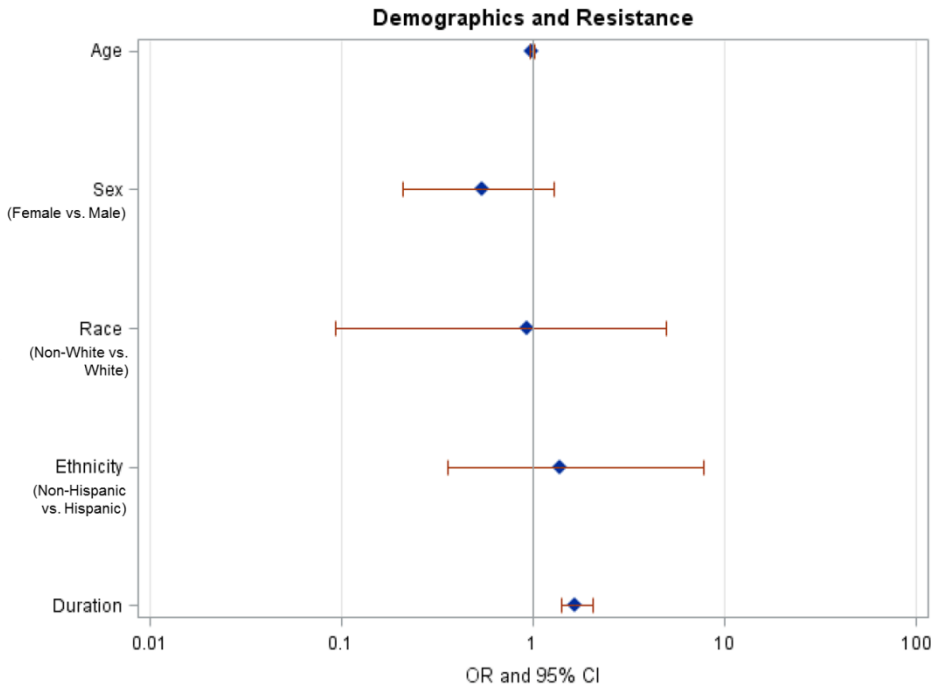
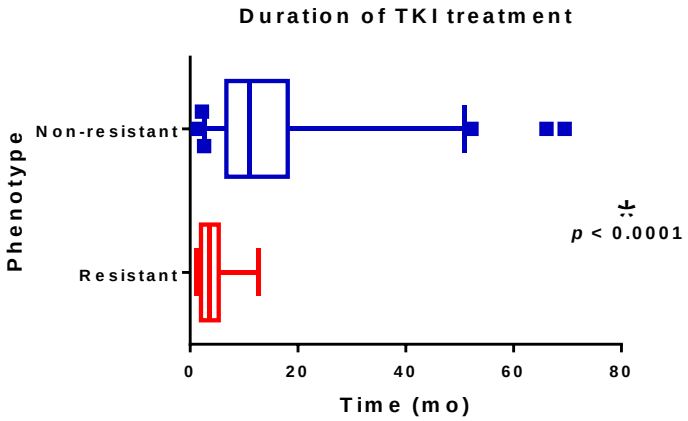


Figure 3.3: Duration of multi-targeted TKI treatment by phenotype. Boxes represent the fiftieth percentiles, with the lines in the middle representing the median values; whiskers represent the 2.5 to 97.5 percentiles.



CHAPTER 4: IDENTIFYING VARIABLES INFLUENCING THE FEASIBILITY OF USING FORMALIN-FIXED PARAFFIN-EMBEDDED TISSUE SAMPLES TO GENERATE WHOLE EXOME SEQUENCING

Summary

Formalin-fixed paraffin-embedded (FFPE) tissue samples represent one of the most potentially valuable resources for clinical research. However, molecular alterations caused by the formalin fixation process, such as DNA crosslinking and degradation, present challenges to optimizing the value of these samples. Despite these challenges, multiple studies have demonstrated that next-generation sequencing of matched FFPE and fresh frozen tissue samples generate highly concordant sequencing data; however, a couple of studies have provided preliminary evidence to suggest that older FFPE samples may not yield quality sequencing results.

We conducted an exploratory study to determine if age of FFPE tumor samples affects ability to generate high quality whole exome sequencing (WES) data. Patient samples were identified from the Total Cancer Care cohort at Moffitt Cancer Center. Patients were included based on cancer diagnosis (sarcoma or renal cell), receipt of a multi-targeted tyrosine kinase inhibitor, and age of FFPE sample available (grouped into 1 to 3, 3 to 5, 5 to 7, 7 to 10, and greater than 10 years old). To maximize data quality, DNA was extracted from FFPE blocks using an FFPE-specific DNA extraction kit and WES was conducted using protocols modified for FFPE samples. Quality of WES was assessed using standard criteria for tumor sequencing, and included fold-coverage, percent of duplicate reads, percent of properly paired paired-end reads, percent of reads that mapped to the reference genome, and transition-to-transversion ratio.

Factors explored as potential predictors of WES success included the age, tissue source, amount of DNA yield, and DNA integrity (DIN) of the FFPE samples.

DNA extraction and sequencing of the 28 selected samples found that 2 (7.1%) failed to generate a sufficient quantity of DNA and 1 (3.6%) had some low-quality WES metrics. Both of the samples that failed to yield sufficient DNA were the only tumors derived from bone tissue. There was no association between the age of FFPE samples and failure to generate quality WES ($p = 0.99$). However, there was an association between DNA yield from FFPE samples and DIN and ability to generate quality WES ($p < 0.001$ and 0.04, respectively). Our results suggest that FFPE tumor samples provide a reasonable, valuable source for retrospective genetic studies regardless of storage time. Rather, factors that may limit usability of samples include amount of DNA yield and potentially DNA integrity or derivation from bone tissue.

Introduction

Formalin fixation with paraffin embedding was first described over 100 years ago, and represents the standard tissue sample preparation method used by pathologists for decades. A major benefit of formalin-fixed paraffin-embedded (FFPE) samples is that they are stable at room temperature, making them easily storable and suitable for large collections of clinical samples associated with historical clinical records. In fact, it has been reported that there are more than a billion FFPE tissue samples archived in hospitals and tissue banks around the world, making FFPE samples one of the most potentially valuable resources for retrospective clinical research.[187]

The potential advantage of FFPE samples in clinical research is challenged by the molecular alterations that result from the formalin fixation and paraffin embedding process. It is

well established that FFPE samples undergo extensive degradation and nucleic acid alterations during preparation. Specifically, the formalin fixation process causes hydrolysis of phosphodiester bonds leading to DNA fragmentation.[188] When performing paired-end sequencing (i.e., DNA sequencing in the forward and reverse direction), DNA fragmentation has potential to result in an excess of overlapping, or duplicate reads. Additionally, the cross-linking of cytosine nucleotides with formalin inhibits DNA polymerase from recognizing the cytosine residues and incorporates adenine in place of guanine, resulting in erroneous C>T or G>A mutations.[189] These artificial mutations as known as sequencing artifacts, and are more common in FFPE samples when compared to fresh frozen (FF) samples.[190]

The advent of next-generation sequencing (NGS) has the potential to overcome the molecular challenges inherent to FFPE samples. NGS is high-throughput, massively parallel, deep sequencing. The power of NGS to analyze small segments of DNA in depth with repetitive coverage minimizes the challenges of fragmentation and mutations introduced by formalin fixation. First, the presence of introduced overlapping reads in FFPE-derived samples will become apparent at high coverage, allowing for duplicate reads to be discarded. Similarly, the sequencing artifacts will be distributed randomly throughout the genome and excessive mismatches within a region will not align properly to the reference genome and will be discarded as erroneous mutation calls. In fact, there have been numerous studies demonstrating high concordance between NGS of matched FF and FFPE tissues. Oh and colleagues conducted whole-exome sequencing (WES) of matched FF and FFPE samples from four cancer patients and found evidence of DNA damage in the FFPE samples, including higher rates of artificial base alterations (mostly C>T or G>A) and higher number of overlapping paired reads (29% vs. 12%); however, after filtering these artifacts and regions of low coverage, high concordance of

mutation calls were reported.[190] In a similar study, Munchel and colleagues conducted targeted and whole genome sequencing of 13 pairs of matched FF and FFPE tissue samples, and found high (>98.9%) concordance of WES base calls and single nucleotide variant calls (>96%) in all matched pairs.[191] Bonfiglio and colleagues reported slightly higher duplicate rates in FFPE samples than FF samples (3.6% vs. 1.8%, respectively), as would be expected, but also observed high (>90%) concordance between single nucleotide variant calls in FF and FFPE-derived tumor samples.[192] Astolfi and colleagues classified FFPE samples as generating low quality or high quality DNA, and found that the sequencing calls from high-quality FFPE samples generated results highly concordant (94-96%) to FF-derived sequencing.[193] These findings emphasize the importance of high-quality DNA and high sequencing coverage to overcome challenges with generating quality sequencing data from FFPE samples.

Limited studies have suggested that age of FFPE samples (i.e., amount of time stored) may be an important factor to consider when using FFPE-derived samples for NGS. A study exploring the effects of formalin fixation on nucleic acids hypothesized that extensive storage of FFPE samples at room temperature may lead to nucleic acid degradation.[188] A comparative study of matched FF/FFPE samples (some from tumor and some from normal tissue) from a range of storage times reported that there was a tendency for DNA degradation and percent of duplicates to increase with storage time.[194] The limiting step for DNA sequencing generation from older FFPE samples was preparation of the library, and library preparation yields were inversely correlated with storage time. Another study demonstrated that older FFPE samples had lower coverage (6% lower per 10 years) and lower average read depth (40x lower per 10 years), but usable NGS data was generated from 90% of samples, regardless of storage time.[195] Therefore, while FFPE samples provide a vast resource for retrospective clinical studies, the usability of all

of the samples, particularly older samples, remains uncertain. We conducted a pilot study to explore the effect of age of FFPE samples on WES quality using the Agilent SureSelect^{XT} Clinical Research Exome kit in conjunction with the newly released FFPE QC kit (Agilent, Santa Clara, CA). Additional factors explored for association with WES quality were FFPE-derived DNA quality and quantity. We hypothesized that older FFPE blocks would result in low-quality WES.

Methods

Patient samples

Patients were identified from the Total Cancer Care (TCC) cohort at Moffitt Cancer Center, an institutional review board (IRB)-approved biobanking protocol in which individuals agree to provide tissue and blood samples for research and to be followed throughout their lifetime.[186] Additional IRB-approval was granted to request tissue samples and conduct this particular study under a de-identified pilot protocol (MCC #50113) using the TCC cohort. Therefore, patient protected health information (PHI) was not accessible for detailed analysis. Individuals eligible for study inclusion were patients consented between January 1, 1994 and October 31, 2015 over the age of 18 years diagnosed with any type of renal carcinoma or sarcoma and treated with a multi-targeted tyrosine kinase inhibitor (axitinib, cabozantinib, pazopanib, regorafenib, sorafenib, sunitinib, or vandetanib). A tumor FFPE sample that could be used for DNA extraction and WES was also required. Eligible patients were identified through collaboration with Moffitt Cancer Center Tissue Core.

For this pilot study, we aimed to include a total of 25 individuals with samples spanning a range of ages and divided by tumor type: age 1-3 years, 3-5 years, 5-7 years, 7-10 years, >10 years, with each category consisting of three renal carcinoma patients and two sarcoma patients

(the most common indications for the multi-targeted TKIs). FFPE samples were diagnostic specimens collected during routine clinical practice at Moffitt Cancer Center. Therefore, the detailed information on sample handling from the time of surgery and sample preparation and specific fixation methods are unavailable; however, this represents the typical situation for research using FFPE-derived samples.

DNA extraction and quantification

DNA was extracted from macrodissected FFPE tissue, quantified using Qubit™ quantification (ThermoFisher Scientific, Waltham, MA), and quality was assessed using a 2200 TapeStation Instrument (Agilent, Santa Clara, CA). One 4 µm section and multiple thick (20 µm) sections were cut from each FFPE block and mounted on glass slides. The number of thick sections cut depended on the target tissue area present on each FFPE block. The thin section was processed for haematoxylin and eosin (H&E) staining and target tissue of the thick sections was used for DNA extraction. A pathologist reviewed the H&E slides and circled the tissue areas containing target tissue to be harvested. The marked H&E slide was used as a template and target tissue was scraped with a razor from glass slides and transferred to an Eppendorf tube. DNA was extracted using a QIAamp DNA FFPE Tissue Kit (QIAGEN, Hilden, Germany) and dissolved in water. DNA was quantified using a NanoDrop (Wilmington, DE) 1000 spectrophotometer, and double-stranded DNA quantity was assessed with Qubit™. The TapeStation was used with Genomic DNA Analysis ScreenTape (Agilent) to assess DNA quality. Quantitative PCR (qPCR) was conducted using an NGS FFPE QC kit (Agilent), which is specifically designed to evaluate the quantity and quality (i.e., DNA integrity) of FFPE-derived DNA. The manufacturer's protocol according to the provided modifications for the SureSelect^{XT} workflow was followed.

Whole exome sequencing

Whole exome sequencing (WES) was performed in order to identify somatic mutations in the coding regions of the human genome. Briefly, 200 ng of DNA, as quantified by qPCR, was used as input for library preparation with the SureSelect^{XT} Reagent Kit (Agilent, Santa Clara, CA). For each tumor DNA sample, a genomic DNA library was constructed according to the SureSelect^{XT} Target Enrichment for Illumina Multiplexed Sequencing (#G7530-90000) protocol (Agilent), including the suggested modifications for FFPE-derived DNA samples (i.e., DNA shearing for 4 min, no dilution of the oligo mix for ligation, and 30 μL of captured DNA used for PCR). The pre-captured library was amplified using 12 PCR cycles, and the size and quality of the library was evaluated using a 2100 BioAnalzyer (Agilent) and Qubit™ quantification. About 500 to 750 ng of pre-captured library was used for hybridization at 65°C for 24 hr. Hybridization and target enrichment were conducted using the SureSelect^{XT} Clinical Research Exome kit (Agilent), which includes all targets of the SureSelect Human All Exon V5 kit with increased coverage at 5000 disease-associated regions (including, but not limited to cancer). The post-captured library was amplified with 10 PCR cycles and evaluated with a 2100 Bioanalyzer (Agilent). The enriched library was quantified using a Library Quantification Kit for NGS (KAPA Biosystems, Wilmington, MA), and samples were diluted to a 4 nM concentration. Denaturation was conducted using NaOH, followed by neutralization with Tris buffer pH 8.5, and samples were diluted in HT1 (hybridization buffer) to a concentration of 20 pM in HT1 buffer. Next, samples were diluted to concentrations between 1.7 pM to 2.2 pM for sequencing with a v2 sequencing reagent kit and a NextSeq 500 desktop sequencer (Illumina, San Diego, CA). Approximately 85 million 75 base paired-end reads were generated for each DNA sample.

Whole exome sequencing quality control

Sequence reads were aligned to the reference human genome (hs37d5) with the Burrows-Wheeler Aligner (BWA),[196] and duplicate identification, insertion/deletion realignment, quality score recalibration, and variant identification were performed with Picard (Broad Institute, http://broadinstitute.github.io/picard/) and the Genome Analysis ToolKit (GATK) v2.2-Lite.[197] Genotypes were determined across all samples at variant positions. Sequence variants were annotated to determine genic context (i.e., non-synonymous, missense, splicing) using ANNOVAR,[198] and summarized using spreadsheets and a genomic data visualization tool, VarSifter.[199] Additional contextual information from other studies was added, including allele frequency from 1000 Genomes[200] and the NHLBI Exome Sequence Project,[201] *in silico* function impact predictions, and observed impacts from databases including ClinVar (NCBI, http://www.ncbi.nlm.nih.gov/clinvar/), the Catalogue of Somatic Mutations in Cancer (COSMIC),[202] and The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/). Somatic mutations were prioritized by excluding variants observed in 1000 Genomes[200] and variants observed at >5% in an internal dataset of adjacent normal (i.e., non-tumor) tissue. Variants with GATK variant quality score recalibration (VQSR) tranche >99.90 or genotype quality (GQ) <15 were excluded.

Data analysis

For this exploratory study, the primary outcome was generation of WES data that passed the quality control filters discussed above (yes/no) and the primary predictor was age group (i.e., 1-3 years, 3-5 years, 5-7 years, 7-10 years, and >10 years) of FFPE blocks. Additional factors considered included DNA integrity number (DIN), a DNA quality metric generated using Agilent TapeStation,[203] DNA yield (amount in µg) from FFPE sample, and source of the DNA

sample. The relationship between age of FFPE sample with DNA yield and DIN was also explored. For this exploratory study, noticeable trends in failed WES were identified and noted.

Nonparametric statistics were used for analysis as to not assume normality of our data. Potential association between DNA yield and DIN with age group of FFPE samples was explored using a Kruskal-Wallis test. The relationship between DNA yield and DIN was tested using linear regression. Values plus or minus more than three standard deviations of the mean were excluded as outliers. Differences in variables between samples that passed WES quality control and those that failed were compared using Mann Whitney U tests for continuous variables. For this exploratory study, a p-value < 0.05 was considered significant. Statistics and figures were generated using GraphPad Prism 6.

Results

Patient population and DNA extraction from FFPE

A total of 28 tumor FFPE samples from the Moffitt Cancer Center Tissue Core were used for DNA extraction (Table 4.1, Appendix 4.1). The final sample population consisted of 6 (21.4%) renal cell carcinoma patients and 22 (78.6%) sarcoma patients; ages of the FFPE blocks received were 1-3 years (8/28, 28.6%), 3-5 years (6/28, 21.4%), 5-7 years (6/28, 21.4%), 7-10 years (4/28, 14.3%), and >10 years (4/28, 14.3%). Two (7%) FFPE samples did not yield sufficient DNA (<80 ng) for library preparation and were excluded from WES. These samples were both sarcomas derived from bone tissue, and one was 1-3 years old while the other was 7-10 years old (Table 4.1).

Whole exome sequencing quality metrics

A total of 26 human tumors underwent WES. The average total number of reads per sample was 1.83×10^8 (95% CI $1.75 \times 10^8 – 1.92 \times 10^8$), with an average of 20% duplicate reads

(Figure 4.1). One sample (#15) had a much higher number of total reads (2.51×10^8) and percent duplicates (60%) than all other samples, raising concern for sequencing quality. After removing duplicates, paired-end reads were properly paired overall (average per sample 94%), and resulting WES aligned well to the human genome, with an average of 98% of reads mapped (Figure 4.1). An average coverage of 151x (95% CI 139.8 – 163.2) per base was achieved; sample #15 had the lowest coverage (82x, Figure 4.2). An average of 74,349 variants were detected per sample, of which 22,993 (31%) were within coding regions of the genome, and 11,590 (16%) were non-synonymous variants. The transition to transversion ratio (Ts/Tv) overall was close to 2 (1.9 ± 0.09), and for the coding region only was closer to 3 (2.7 ± 0.08), as would be expected.[197]

Factors affecting whole exome sequencing quality

We explored multiple factors that could impact the ability to generate good quality WES from FFPE samples, including age of the sample, DNA yield, DNA quality as measured by DNA integrity number (DIN), and tissue source of the sample (Table 4.1). There was no significant association between age group of FFPE samples and DNA yield ($H = 4.54$, 4 df, $p = 0.34$) or DIN ($H = 4.67$, 4 df, $p = 0.32$) (Figure 4.3). Notable trends included the observation that only samples under the age of 7 yr yielded greater than 10 µg of DNA and only the FFPE samples *less* than 7 yr old had DIN readings below the limit of detection (Figure 4.3). After removing one outlier from analysis (DNA yield = 40.55 µg, > 3 SD of the mean) there was no relationship between the DNA yield and DIN ($r^2 = 0.12$, $p = 0.08$).

There were a total of 3/28 (10.7%) samples that failed WES: two failed during library preparation and one (#15) resulted in potentially concerning WES data (high percent duplicates and low coverage, Figure 4.1 and Figure 4.2). There was no difference in age between the

samples that failed WES quality control and those that passed (U = 26.5, p = 0.99). However, the failed and low quality samples had significantly lower DNA extraction yields than the samples that passed quality control (median of failed samples: 0.09 µg, median of passed samples: 4.24 µg; U = 0, p = 0.0006) (Figure 4.4). There was also a significant association between DIN and WES failure (median of failed samples: 0 µg, median of passed samples: 2.7 µg; U = 7.5, p = 0.04), although 1/3 of the failed samples had a reasonable DIN (2.1) and 6/25 (24%) of the passed samples had a DIN ≤ 2.1(4 were below the limit of detection) (Figure 4.4).

Discussion

We conducted a pilot study to explore the effect of age of FFPE samples on the ability to generate good, usable, quality WES. We also explored other potential factors of variability in success of WES, including DNA quantity and quality, as well as the tissue source of DNA. Unexpectedly, we observed a low WES failure rate in our cohort, which did not appear to be associated with age of the FFPE samples. There was also no clear association between the age of the FFPE samples and the quantity or quality of DNA yield. Interestingly, the two failed samples were from a range of age groups and had varying DINs, but both were derived from bone tissue. It is established that DNA extraction from bone tissue is challenging,[204] but to our knowledge, the ability or challenges of extracting DNA from bone FFPE samples has yet to be reported.

We successfully generated high quality WES data from the 26 samples that generated sufficient DNA yields. Samples generated, on average, over 180 million paired-end reads, with a low frequency of duplicate reads, which resulted in high coverage of the genome that mapped well to the human reference genome. One sample (#15) generated possibly concerning WES data, with a much higher percentage of duplicate reads than the other samples and the lowest average coverage; however, after discarding the duplicate reads the percentage of properly paired

paired-end reads and percentage of reads that properly mapped to the human reference genome remained high. Of note, this FFPE sample also had a low DNA extraction yield, an undetectable DIN, and was derived from a soft tissue tumor (3-5 yr old). All other samples resulted in high quality WES data that met our predefined quality control thresholds. A notable limitation of this study is the age cutoff of samples. Due to the drug approval timeline of multi-targeted tyrosine kinase inhibitors, the oldest sample included in our study was 16 yr old. Therefore, we are unable to determine if there is an older age, perhaps over 20 yr, that would indefinitely result in low DNA yield and poor sequencing output.

In conclusion, we found no association between age of FFPE sample and ability to generate quality WES data. Factors that may be important to consider before conducting WES using FFPE samples include amount of DNA yield (our two failed samples and one borderline sample all yielded less than 0.2 μg of DNA) and tissue source of FFPE sample, since both samples that yielded insufficient DNA were derived from bone FFPE blocks. This study confirms our methods for generating high quality, usable, WES data from tumor FFPE blocks stored for a range of timeframes.

Tables and Figures

Table 4.1: FFPE sample information including extracted DNA quantity and quality criteria. Sample number "NA" corresponds to FFPE samples that did not yield sufficient DNA for library preparation. WES: Whole exome sequencing; DIN: DNA integrity number.

WES sample number	Diagnosis	Tissue Type	Age of FFPE (yrs)	Qubit dsDNA (µg)	DIN	Pass/Fail WES
1	Sarcoma	Kidney	5-7	2.60	3.10	Pass
2	Sarcoma	Rectum-Anus	>10	9.85	2.60	Pass
3	Sarcoma	Small Intestine	>10	2.96	2.60	Pass
4	Sarcoma	Stomach	>10	4.50	2.70	Pass
5	Sarcoma	Skin	>10	2.19	2.20	Pass
6	Sarcoma	Soft Tissue	7-10	1.96	2.80	Pass
7	Sarcoma	Breast	7-10	0.24	1.80	Pass
8	Sarcoma	Soft Tissue	7-10	3.87	2.40	Pass
NA	Sarcoma	Bone	7-10	0.16	2.10	Fail (insufficient DNA)
9	Sarcoma	Abdomen	5-7	1.55	2.10	Pass
10	Sarcoma	Soft Tissue	5-7	40.55	3.90	Pass
11	Renal cell carcinoma	Kidney	5-7	5.85	3.20	Pass
12	Sarcoma	Soft Tissue	5-7	0.21	-	Pass
13	Sarcoma	Soft Tissue	5-7	0.82	2.90	Pass
14	Renal cell carcinoma	Kidney	3-5	10.15	3.10	Pass
15	Sarcoma	Soft Tissue	3-5	0.09	-	Potential fail (WES QC)
16	Sarcoma	Retroperitoneum	3-5	10.95	2.70	Pass
17	Sarcoma	Kidney	3-5	19.35	3.60	Pass

18	Sarcoma	Bone	3-5	11.35	2.90	Pass
19	Sarcoma	Thoracic	3-5	1.70	3.70	Pass
20	Sarcoma	Soft Tissue	1-3	7.90	-	Pass
21	Sarcoma	Soft Tissue	1-3	2.87	2.30	Pass
22	Renal cell carcinoma	Kidney	1-3	11.30	2.50	Pass
23	Sarcoma	Bone	1-3	13.90	3.00	Pass
NA	Sarcoma	Bone	1-3	0.04	-	Fail (insufficient DNA)
24	Renal cell carcinoma	Kidney	1-3	4.24	-	Pass
25	Renal cell carcinoma	Kidney	1-3	3.90	4.60	Pass
26	Renal cell carcinoma	Kidney	1-3	4.57	-	Pass

- corresponds to samples with a DIN below the limit of detection

Figure 4.1: Summary of whole exome sequencing sample reads. Information is presented for each sample for which whole exome sequencing was generated, and includes the total number of reads (blue circle), the percentage of duplicate reads (red square), the percent of reads mapped to the reference human genome (hs37d5) (green triangle), and the percent properly paired-end reads (purple upside-down triangle). The left y-axis represents the total reads, and the right y-axis represents all other values, presented as percents.

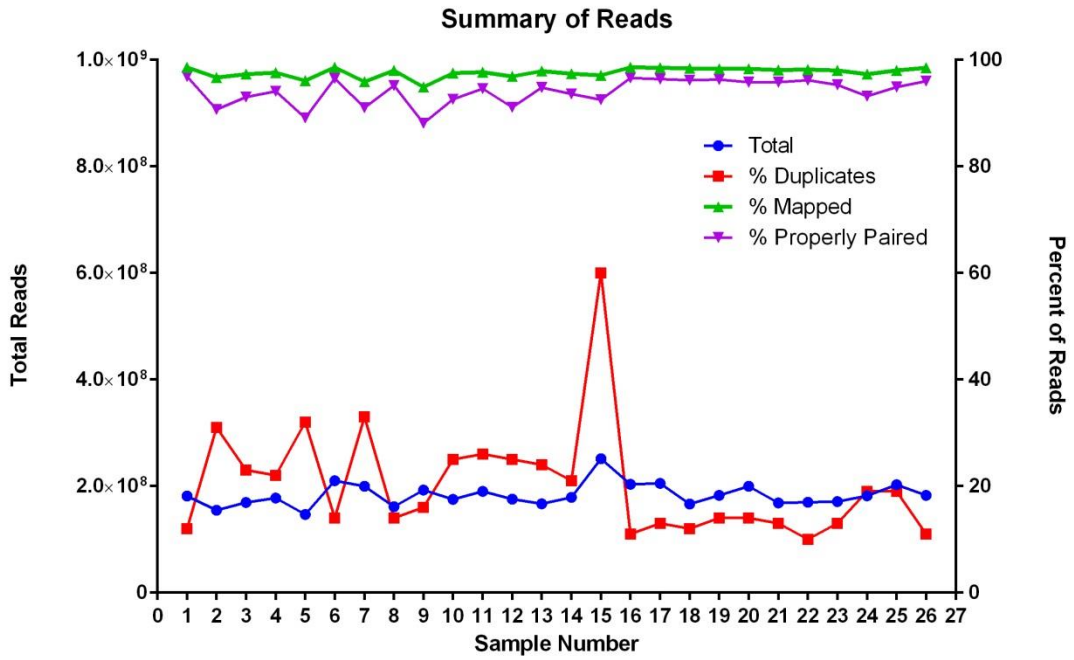


Figure 4.2: Average sequencing coverage depth per base by sample. Dashed line at 100x represents threshold for high quality yield from sequencing of a tumor sample.

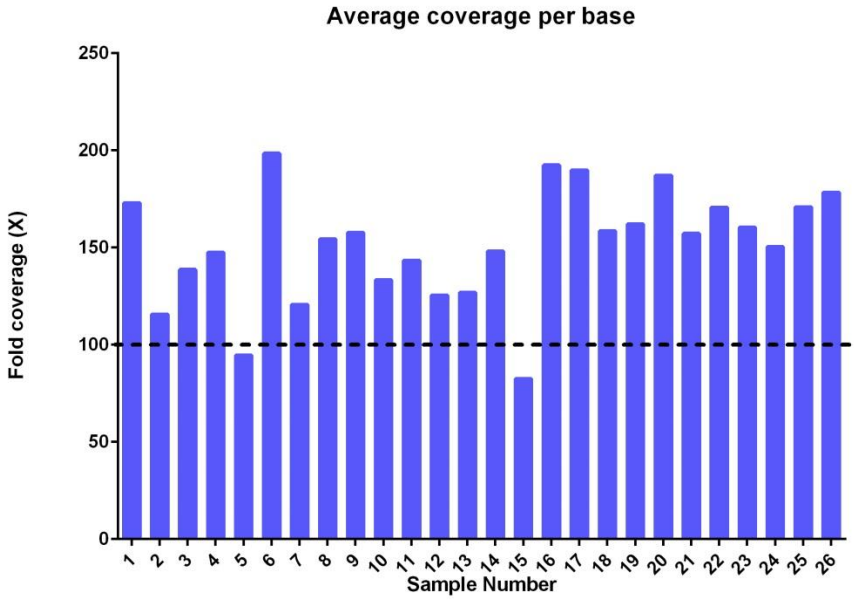


Figure 4.3: Effect of age on FFPE DNA yield and DNA integrity. Graphs display median and interquartile ranges for age groups, with individual measurements also shown. Exes (×) represent samples that failed library preparation (n=2) or some whole exome sequencing quality metrics (n=1). DIN: DNA integrity number.

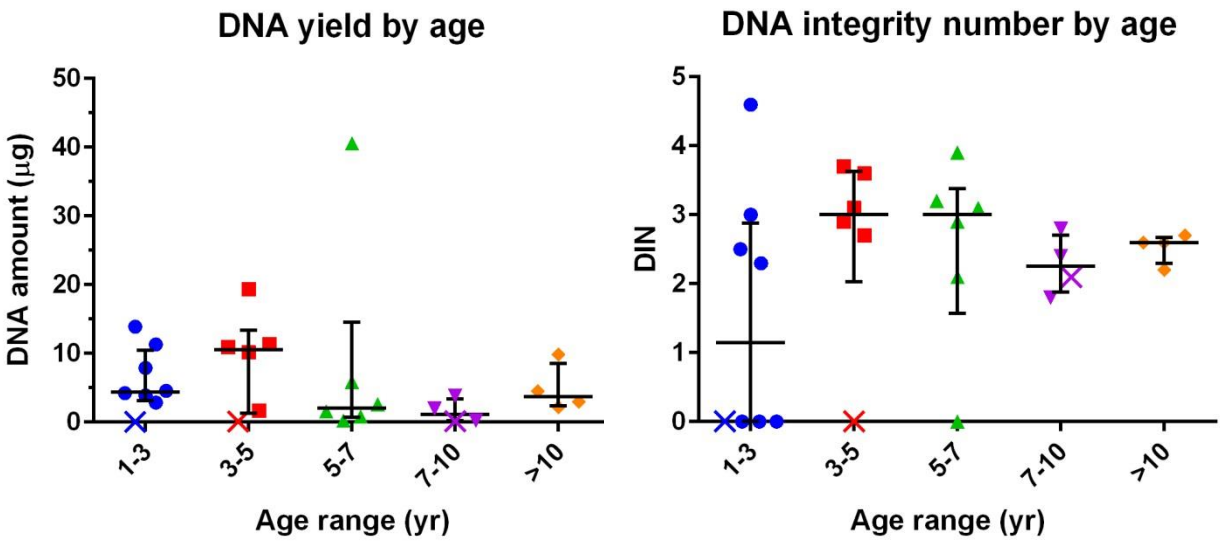
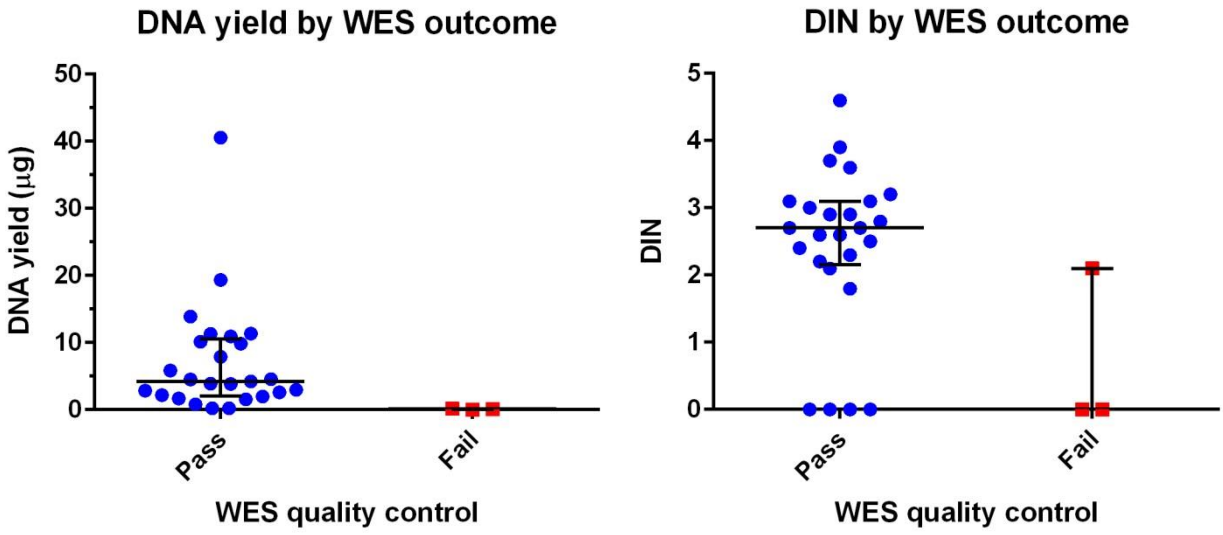


Figure 4.4: DNA extraction yield and DNA integrity (DIN) grouped by success of whole exome sequencing (WES). Graphs display median and interquartile ranges for age groups, with individual measurements also shown.



CHAPTER 5: IDENTIFYING GENETIC PREDICTORS OF INTRINSIC RESISTANCE TO MULTI-TARGETED

Summary

Multi-targeted tyrosine kinase inhibitor (TKIs) are widely prescribed anticancer agents that are effective in a range of solid and hematologic malignancies, including renal cell carcinoma, sarcomas, and pancreatic neuroendocrine tumors, amongst others. The broad FDA-approved indications and efficacy of multi-targeted TKIs suggest shared molecular drug targets across cancer types. However, irrespective of tumor type, approximately 20% to 30% of patients treated with multi-targeted TKIs derive no benefit, with progressive disease as a best response, demonstrating intrinsic resistance. We hypothesized that there are genetic alterations, shared across tumor types, which are associated with intrinsic resistance to the multi-targeted TKIs. We conducted a retrospective cohort study to identify tumor (somatic) point mutations and copy number alterations that are associated with resistance to multi-targeted TKIs. We conducted combined analysis to develop an algorithm that to differentiate intrinsically resistant from non-resistant patients based on molecular testing results. Patients were identified from the Total Cancer Care cohort at Moffitt Cancer Center based on previous treatment with a multi-targeted TKI for any solid tumor indication. Patients were classified as intrinsically resistant or non-resistant based on detailed medical chart reviews. Intrinsic resistance was defined as progressive disease at first imaging follow-up and non-resistance was defined as no evidence of progressive disease (i.e., partial response, stable disease, or mixed response) at first imaging follow-up. Next-generation sequencing (targeted exome capture) and whole genome copy number variation (CNV) analysis of tumor samples was performed to identify somatic alterations in resistant and

non-resistant individuals. We observed a 30% intrinsic resistance rate in our cohort. A total of 243 candidate genes related to cancer pathogenesis or anticancer drug response were included in analyses. Resistant individuals more commonly harbored somatic point mutations in *NTRK1*, *KDR*, *TGFBR2*, and *PTPN11* (uncorrected p-value < 0.1), and CNV in *CDK4*, *CDKN2B*, and *ERBB2* was associated with resistance at an exploratory cut-off (FDR-corrected p-value < 0.3). Combined analysis using random forest resulted in a decision tree that included many of the same hits from individual analyses. CNV in *CDK4* and *CDKN2B* were identified as the most important features and, alone, could be used to differentiate 55% of individuals as resistant or non-resistant, thus implicating the cyclin D pathway as an important factor in resistance to multi-targeted TKIs. Despite insufficient statistical power, we identified candidate genes with strong biologic plausibility of conferring resistance to multi-targeted TKIs. Independent validation is warranted before implementing these results in clinical practice.

Introduction

Increasing evidence supports the classification of tumors based on genetic and molecular characteristics rather than site of origin, which has translated into the ability to predict drug response utilizing tumor genetics rather than tumor type (i.e., histology or site of origin) in some instances. For example, using multiple genetic platforms, The Cancer Genome Atlas Research Network defined four molecular subtypes of breast cancer (Luminal A, Luminal B, HER2-enriched, and Basal-like) and found that one subtype (Basal-like) is more similar to serous ovarian cancer than to other breast cancers.[205] Further analysis showed that, due to their molecular similarities, Basal-like breast cancers and serous ovarian cancers are likely susceptible to similar targeted treatments. In another multiplatform analysis of twelve tissue-defined cancer

types, eleven major subtypes were identified, with only five subtypes corresponding to their tissue of origin, and the remaining six subtypes being shared by distinct cancer types (e.g., lung squamous, head and neck, and some bladder cancers fell into a single subtype).[206] Studies such as these have influenced clinical cancer drug development. The classification and treatment of tumors based on molecular alterations is currently being studied through the use of basket trials, such as NCI-MATCH and NCI-MPACT, which randomize patients to an individualized targeted therapy arm or a non-pathway-specific arm independent of tumor histology.[207] We hypothesize that genetic alterations within tumors, regardless of site of origin or histology, can be used as a biomarker of response to targeted anticancer therapies.

The broad efficacy of multi-targeted tyrosine kinase inhibitors (TKIs) across tumor types suggests similarities in the genetics of the tumors they are used to treat. The multi-targeted TKIs (i.e., axitinib, cabozantinib, pazopanib, regorafenib, sorafenib, sunitinib, and vandetanib) currently have FDA-approved indications in seven histological tumor types and are also used off-label in additional solid and hematologic malignancies. While the clinical trials of these agents demonstrated overall efficacy, a substantial minority of individuals never responded to therapy. In the pivotal phase III clinical trials that led to approval of each of these agents, approximately 20 to 30% of patients showed a best response of progressive disease, demonstrating intrinsic resistance.[104,134-140] Similarly, in a systematic review analyzing clinical data from twelve medical centers, Heng and colleagues observed that 26% of 1,056 renal cell carcinoma patients treated with multi-targeted TKIs demonstrated primary, or intrinsic, resistance.[141] These patients received no benefit from the multi-targeted TKIs, but were put at risk for serious, potentially fatal, adverse events while delaying use of other potentially effective treatments. Furthermore, it has been demonstrated that patients with intrinsic resistance to multi-

targeted TKIs have poorer clinical outcomes with subsequent lines of therapies.[208] In an exploratory study examining demographic and clinical factors that may contribute to intrinsic resistance to multi-targeted TKIs across tumor types (*See Chapter 3*), we observed an intrinsic resistance rate of 21%, which also correlated well with that observed in clinical trials. Most interestingly, we did not identify any demographic or clinical factors (e.g., tumor type or drug received) that were robustly associated with intrinsic resistance. Results of this study further support the hypothesis that genetic factors may be important in predicting intrinsic resistance to the multi-targeted TKIs.

Targeted cancer therapies, such as the TKIs, function by inhibiting molecular targets involved in cancer cell proliferation, growth, and survival, making the genetics of the tumor (i.e., somatic genetics) critical in therapeutic response. Elegant studies utilizing tissue from patients treated with TKIs have demonstrated that tumors can acquire mutations or gene amplifications that confer secondary resistance.[209-211] However, potential mechanisms of intrinsic resistance to multi-targeted TKIs are unexplored.[212-214] Possible causes of intrinsic resistance include mutations or alterations in expression of drug targets or important oncogenic pathway genes within a tumor. The single-targeted epidermal growth factor receptor (EGFR)-TKI, erlotinib, and the monoclonal antibody EGFR inhibitors, cetuximab and panitumumab, provide an example of the importance genetic variation can play in intrinsic resistance. Post-FDA approval, it was discovered that mutations in the *RAS* gene, which is downstream of the EGFR receptor, confer intrinsic resistance to EGFR-targeted inhibitors.[47,215,216] *EGFR* and *KRAS* mutation status are now routinely tested in clinical practice before prescribing EGFR inhibitors. Recently, Carter and colleagues utilized circulating tumor cells to identify copy number alterations in small cell lung cancer patients and demonstrated that the copy number alterations in individuals with intrinsic

resistance were unique from those with acquired resistance, providing evidence that intrinsic resistance is a distinct molecular phenomenon.[217]

Preemptive identification of patients intrinsically resistant to multi-targeted TKIs is critical to avoid unnecessary toxicity risk and guide treatment selection toward other potentially effective therapies. We hypothesize that there are somatic alterations in the molecular targets or pathways inhibited by multi-targeted TKIs that can be used to predict intrinsic resistance. We utilized an extensively annotated tissue biobank to conduct a retrospective candidate gene study aimed at identifying somatic point mutations and copy number alterations associated with intrinsic resistance to multi-targeted TKIs.

Methods

Patient Population

Patients were identified from the Total Cancer Care (TCC) cohort at Moffitt Cancer Center, an institutional review board (IRB)-approved biobanking protocol in which individuals agree to provide tissue and blood samples for research and to be followed throughout their lifetime.[186] Additional Moffitt Cancer Center Scientific Review Committee and IRB-approvals (MCC #18318 and #18790) were granted to request tissue samples and conduct this particular study using the TCC cohort. Individuals eligible for study inclusion were patients consented between January 1, 1994 and December 31, 2015 over the age of 18 years diagnosed with any type of solid tumor and treated with a multi-targeted tyrosine kinase inhibitor (axitinib, cabozantinib, pazopanib, regorafenib, sorafenib, sunitinib, or vandetanib). Individuals were included if they had targeted exome sequencing or whole exome sequencing (WES) data available through the TCC protocol or if a tumor FFPE sample was available that could be used for DNA extraction and WES. Exclusion criteria included individuals treated with a multi-

targeted TKI for a hematologic malignancy and individuals whose tumor FFPE sample(s) were collected after more than three drug regimens post-multi-targeted TKI administration, since the genetics of those tumors may not be representative of the tumor treated with multi-targeted TKI.

Phenotyping

Data was initially abstracted in a standardized manner from the TCC biorepository system (TransMed, Cupertino, CA). Data abstracted included demographic variables (date of birth, gender, race, and ethnicity) as well as clinical information [medical record numbers (MRNs), date of diagnosis, primary site at diagnosis, histology, first course of treatment, multi-targeted TKI received, start and stop date of TKI, and date of death or last follow-up]. Sources for these data were the Florida Cancer Registry and electronic medical records. Information abstracted from TransMed was used to guide manual chart reviews.

Independent manual chart reviews were performed by two clinicians and researchers using the patients' MRNs. Chart reviews included the validation of information generated using TransMed abstraction and manual review of patients' clinical notes and imagining (PET/CT/MRI) results before and after TKI administration. Additional information collected included radiologists' and clinicians' impressions of disease before and after TKI initiation (e.g., stable vs. progressive vs. responding) and time to drug change or discontinuation. Based on these data, reviewers documented their individual impressions of patients as either "resistant" or "non-resistant". Phenotype classifications were then compared and a third reviewer, a physician experienced with TKI prescribing and follow-up, was available for adjudication if necessary.

Physicians' recommendations at first imaging follow-up (generally 2-3 months post-initiation) were used to identify patients intrinsically resistant to multi-targeted TKIs. The decision to stop TKI therapy due to cancer progression was classified as "intrinsic resistance".

Patients who continued TKI therapy after first follow-up due to response, stable disease, or mixed response, and patients who met these criteria but stopped therapy due to side effects were classified as "non-resistant".

Next-generation sequencing and copy number variation

Whole exome sequencing (WES) was performed in order to identify somatic mutations in the coding regions of the human genome. Briefly, 200 ng of DNA, as quantified by qPCR, was used as input for library preparation with the SureSelect^{XT} Reagent Kit (Agilent, Santa Clara, CA). For each tumor DNA sample, a genomic DNA library was constructed according to the SureSelect^{XT} Target Enrichment for Illumina Multiplexed Sequencing (#G7530-90000) protocol (Agilent), including the suggested modifications for FFPE-derived DNA samples. The pre-captured library was amplified, and the size and quality of the library was evaluated using a 2100 BioAnalzyer (Agilent) and Qubit™ quantification (Thermo Fisher Scientific, Waltham, MA). Approximately 500 to 750 ng of pre-captured library was used for hybridization at 65°C for 24 hr. Hybridization and target enrichment were conducted using the SureSelect^{XT} Clinical Research Exome kit (Agilent). The post-captured library was amplified and evaluated with a 2100 Bioanalyzer (Agilent). The enriched library was then quantified using a Library Quantification Kit for NGS (KAPA Biosystems, Wilmington, MA), and samples were diluted to a 4 nM concentration. Denaturation was conducted using NaOH, followed by neutralization with Tris buffer pH 8.5, and samples were diluted to a concentration of 20 pM in HT1. Next, samples were diluted to concentrations between 1.7 pM to 2.2 pM for sequencing with a v2 sequencing reagent kit and a NextSeq 500 desktop sequencer (Illumina, San Diego, CA). Approximately 85 million 75 base paired-end reads were generated for each DNA sample.

Individuals with targeted gene sequencing data available had sequencing performed

previously through collaboration with the Beijing Genomics Institute (Shenzhen, China). Briefly, tumor samples underwent targeted gene sequencing using a custom SureSelect platform (Agilent, Santa Clara, CA) targeting 1,321 cancer-related genes and 2 x 90bp massively parallel sequencing using a Genome Analyzer IIx (Illumina, San Diego, CA). In order to identify whole genome copy number variation (CNV) and loss-of-heterozygosity, the OncoScan® FFPE Assay Kit (Affymetrix, Santa Clara, CA) was used according to the manufacturer's protocol, with an input of 80 ng of FFPE-extracted DNA.

Candidate gene selection

To decrease multiple comparison correction and increase the likelihood of identifying biologically plausible associations, we utilized a candidate gene approach for analysis. Candidate genes included genes known to be important in solid tumor biology and anticancer drug response and were selected utilizing the overlapping genes reported on a validated clinical cancer genetic testing platform (FoundationOne®, Foundation Medicine, Cambridge, MA) and those captured with the targeted sequencing platform designed specifically for the TCC study cohort. A list of the candidate genes is provided in Appendix 5.1.

Data quality control and variant detection

Whole exome sequencing reads were aligned to the reference human genome (hs37d5) with the Burrows-Wheeler Aligner (BWA),[196] and duplicate identification, insertion/deletion realignment, quality score recalibration, and variant identification were performed with Picard (Broad Institute, http://broadinstitute.github.io/picard/) and the Genome Analysis ToolKit (GATK) v2.2-Lite.[197] Genotypes were determined across all samples at variant positions. Sequence variants were annotated to determine genic context (i.e., non-synonymous, missense, splicing) using ANNOVAR,[198] and summarized using spreadsheets and a genomic data

visualization tool, VarSifter.[199] Additional contextual information from other studies was added, including allele frequency from 1000 Genomes[200] and the NHLBI Exome Sequence Project,[201] *in silico* function impact predictions (PolyPhen and SIFT), and observed impacts from databases including ClinVar (NCBI, http://www.ncbi.nlm.nih.gov/clinvar/), the Catalogue of Somatic Mutations in Cancer (COSMIC),[202] and The Cancer Genome Atlas (TCGA, https://cancergenome.nih.gov/). Somatic mutations were prioritized by excluding variants observed in 1000 Genomes[200] and variants observed at >5% in an internal dataset of adjacent normal (i.e., non-tumor) tissue. Variants with GATK variant quality score recalibration (VQSR) tranche > 99.9 or genotype quality (GQ) < 15 were excluded.

The CNV data generated was analyzed using Nexus Copy Number™ 6.0 software (BioDiscovery, El Segundo, CA) utilizing the TuScan™ algorithm, specifically designed for OncoScan® FFPE Assay data.[218] The estimated CNV regions were annotated with the reference human genome (hg19) and the evaluation of array performance was measured using default criteria (MAPD $\leq$ 0.3 and ndSNPQC $\geq$ 26). Plots of whole genome CNVs and minor allele frequencies (BAFs) were generated for each individual. Briefly, the BAF was calculated as the count of minor (B) alleles (A/T) divided by the total count of major (A) (G/C) and minor (B) alleles.[219] Using the TuScan™ algorithm the average CNV of all cells within each sample was generated. Genes with 70% or greater overlap in copy number abberated regions were classified as being altered. Copy number gains greater than seven and homozygous losses were considered potentially clinically significant, as is standard in clinical tumor testing, and included in analyses.

Data analysis

Patients were classified into one of two cohorts: (1) resistant or (2) non-resistant using the phenotyping methods described above. Descriptive statistics were used to summarize

demographic and clinical characteristics of patients included. Means, standard deviations and ranges were calculated for continuous variables, and frequencies and percentages were generated for categorical variables. As a conservative approach and where applicable, non-parametric statistical tests were implemented to avoid assuming the data was normally distributed. Demographic and clinical characteristic comparisons between resistant and non-resistant patients were performed using two-tailed Fisher's exact tests for categorical variables and the Mann-Whitney U test for continuous variables. Batch effects between the two sequencing methods were detected using a two-tailed Fisher's exact test and, when comparing between sequencing platforms, mutated genes with an FDR-corrected p-value < 0.05 were excluded from further analyses. The two-tailed Fisher's exact test was used to test the statistical significance of mutated candidate genes identified in resistant and non-resistant patients. An FDR-corrected p-value < 0.1 was considered statistically significant, and an uncorrected p-value < 0.10 was significant for exploratory associations. Cytoscape (v.3.4.0) Enrichment Map[220] was utilized to conduct gene set enrichment of top gene hits and the Genomics in Drug Sensitivity in Cancer database[221] was utilized to explore *in vivo*-derived predictions of sensitivity to multi-targeted TKIs based on mutation status of the top hits.

For CNV analysis, copy number gains and losses were grouped and presence of copy number aberrations between resistant and non-resistant individuals were arranged in contingency tables and analyzed using a two-sided Fisher's exact test. FDR-corrected p-values < 0.1 were considered statistically significant, and a threshold of FDR-corrected p < 0.3 was used for exploratory analysis.[222,223] Cytoscape (v.3.4.0) Enrichment Map[220] was utilized to conduct gene set enrichment of top gene hits.

Statistical analysis was conducted using the open-source, statistical programming

language, R (version 3.3.2).[224] CNV and WES data were combined to build a model leveraging both technologies. CNVs were annotated as 1, 0, -1 for gain, no change, or loss, respectively, based on the criteria described above. Subjects with > 20% missing data and genes with > 5% missing data were excluded from further analysis. Remaining missing genes were imputed using the mean of the study cohort. Subsequently, feature selection, via a random forest model was constructed using 2000 trees and 12 features at each split.[225] CNV and WES features with a mean decrease in Gini score > 0.2 were selected for inclusion in the decision tree classification model using recursive partitioning trees.[226] Figures were generated using R (version 3.3.2) and GraphPad Prism 6.

Results

Patient population and phenotypes

A total of 50 unique patients were included in this study (Table 5.1). The average age was 59 years old and the majority of individuals were white (86%) non-Hispanic (86%) males (72%). The most common tumor types being treated were sarcoma (52%) and renal cell carcinoma (36%), and the majority of patients received pazopanib (42%), followed by sorafenib (30%) and sunitinib (26%). One patient was treated with regorafenib. Of the 50 patients included, 11 (22%) were classified as resistant, 26 (52%) as non-resistant, and 13 had unclassifiable responses. This corresponded to an overall resistance rate of 30.5% (11/36) observed in our cohort. There were no statistically significant differences in the demographic and clinical characteristics of resistant and non-resistant patients; however, non-resistance trended toward being more common in renal cell carcinoma patients (Table 5.1). Resistant patients discontinued multi-targeted TKIs significantly sooner than non-resistant patients ($U = 14.5$, $p < 0.0001$, Figure 5.1).

Next-generation sequencing results

A total of 24 samples underwent targeted exome sequencing under the TCC protocol. The target region was 1,321 genes covering 3.8 Mb. The median number of reads aligning per samples was 15,283,830. Median read depth of coverage was 141x. A median of 93.7% of coding bases were covered ≥ 10x across samples. For the 25 tumor samples that underwent whole exome sequencing (WES), an average coverage of 151x (95% CI 140 - 163) per base was achieved. The average total number of reads per sample was 1.83×10^8 (95% CI 1.75×10^8 - 1.92×10^8), with an average of 20% duplicate reads. After removing duplicates, paired-end reads were properly paired overall (average per sample 94%), and resulting WES aligned well to the human genome, with an average of 98% of reads mapped. An average of 74,349 variants were detected per sample, of which 22,993 (31%) were within coding regions of the genome, and 11,590 (16%) were non-synonymous variants. There were five genes (*CDK12*, *FGFR4*, *MLL2*, *LRP1B*, and *ARAF*) with significant variation by sequencing batch (FDR-corrected p-values all < 0.005), warranting exclusion from further analyses.

We identified four genes (*NTRK1*, *KDR*, *TGFBR2*, and *PTPN11*) more commonly mutated in resistant patients than non-resistant patients (Figure 5.2). The finding with the lowest p-value was *NTRK1*, in which 3 (30%) resistant patients carried somatic coding mutations (2 point mutations and 1 splice site variant) versus zero non-resistant patients (p = 0.02). Nonsynonymous coding mutations in *KDR*, *PTPN11*, and *TGFBR2* were present in 2 (20%) resistant patients and zero non-resistant patients (p = 0.08). Altogether, 55% of resistant patients harbored mutations in one or more of these genes, while zero non-resistant patients carried mutations in these genes (Fisher's exact p-value = 0.0002, Figure 5.3). Gene set enrichment identified trends of receptor binding, activity, protein kinase, tyrosine, and transmembrane within

the top gene hits. Of the four top hits, *KDR* and *PTPN11* were included in screening 1,001 cancer cell lines in the Genomics in Drug Sensitivity in Cancer database.[227,228] Presence of *KDR* mutations was consistently predicted to confer resistance to multi-targeted TKIs, while presence of *PTPN11* mutations tended to predict sensitivity to multi-targeted TKIs (Appendix 5.2).

Copy number variation results

Copy number variation (CNV) data was generated for 29 individuals, of which 8 (27.6%) were classified as resistant. All samples resulted in data that met pre-specified quality control criteria. Individuals exhibited a diverse range of copy number aberrations, with some individuals demonstrating much more genomic instability than others (Appendix 5.3). A total of 55 (22.6%) genes harbored copy number alterations that met specified filtering criteria (Figure 5.4). No genes met the FDR-corrected significance level, however, three genes (*CDK4*, *CDKN2B*, and *ERBB2*) met the exploratory cut-off (FDR-corrected p = 0.28 for each). CNVs in *CDKN2B* were only observed in resistant patients, while CNVs in *CDK4* and *ERBB2* were less common in resistant patients (only non-resistant patients harbored CNVs in *ERBB2*). All of the CNVs in *CDKN2B* and *ERBB2* were homozygous losses, and the majority (14/16, 87.5%) of aberrations in *CDK4* were also losses. Gene set enrichment identified cancer pathways, cyclin, and kinase as network trends between the three gene hits for CNV.

Decision tree for combined data

The most informative CNV and next-generation sequencing features from the random forest classification model were used to generate a decision tree for identifying resistant individuals. After quality-control, data for the 29 individuals with sequencing and CNV results were used in the construction of the final tree. Five genes (*CDKN2B*, *CDK4*, *TGFBR2*, *EPHA3*, and *TNFAIP3*) were identified as important for differentiating resistant from non-resistant

individuals and were incorporated into the final decision tree (Figure 5.5). CNV in *CDKN2B* and *CDK4* were most informative and explained responses for 55% (16/29) of the population. Interestingly, in measuring the importance of variables using the mean decrease in Gini score, all gene hits from individual sequencing (*NTRK1*, *KDR*, *TGFBR2*, and *PTPN11*) and CNV (*CDK4*, *CDKN2B*, and *ERBB2*) analysis were identified as being amongst the most informative variables (Figure 5.5, Appendix 5.4). The decision tree model resulted in a high sensitivity and specificity for differentiating resistant individuals (0.75 and 1, respectively; balance accuracy 0.88); however, leave-one-out cross validation resulted in a lower sensitivity and specificity (0.25 and 0.95, respectively; balance accuracy 0.6). It further suggested that having the identified combination of mutations increases one's odds of being resistant (OR 6.67, insufficient power).

Discussion

We conducted a retrospective candidate gene study to identify somatic point mutations and copy number alterations associated with intrinsic resistance to multi-targeted TKIs. Using next-generation sequencing we identified four genes commonly mutated in resistant patients, but not mutated in non-resistant patients: *NTRK1*, *KDR*, *TGFBR2*, and *PTPN11*. Interestingly, three of the four top hits (*TGFR*, *KDR*, and *NTRK1*) encode transmembrane protein kinases that are known targets of multi-targeted TKIs. The final gene, *PTPN11*, encodes SHP2, a tyrosine phosphatase that mediates signaling of oncogenic tyrosine kinases, such as Ras-ERK-AKT signaling pathways.

All four of the top gene hits from sequencing analysis are known to be mutated in cancer patients and have some literature suggesting possible associations with resistance and/or prognosis. *KDR* encodes the vascular endothelial growth receptor 2 (VEGFR2), a tyrosine kinase that mediates VEGF-induced endothelial proliferation, survival, and migration. *KDR* is

commonly mutated across cancer types, and is one of the primary targets of the multi-targeted TKIs, with up to eighty percent of activity being inhibited by TKIs.[133] Therefore, mutations in the gene encoding VEGFR2 represent a plausible mechanism of resistance to these agents. In fact, escape from VEGFR2 signaling dependency has been proposed as a mechanism of acquired resistance to the multi-targeted TKIs.[229] In a recent retrospective analysis of archived renal cell carcinoma patients treated with sunitinib, Stubbs and colleagues found no association between *KDR* expression and overall or progression-free survival.[230] However, a retrospective study of 275 sarcoma patients identified a significant correlation between high VEGFR2 protein expression (measured using immunohistochemistry) and decreased patient survival ($p < 0.001$).[231] The most common *NTRK1* alterations observed in cancer are gene fusions, however, point mutations have also been reported in numerous solid tumors.[232,233] Multiple studies have linked *NTRK1* overexpression to tumor progression and poor outcomes in solid cancers,[234-236] and *NTRK1* mutations are known to confer acquired resistance to NTRK inhibitors.[237] Somatic mutations of *TGFBR2* are commonly observed across solid tumor types.[232,233] The majority of studies exploring the clinical significance of *TGFBR2* mutations are in the context of breast cancer, where it has been demonstrated that high expression of *TGFBR2* is associated with tumor metastasis and response to chemotherapy.[208,238,239] Associations between somatic *TGFBR2* alterations and cancer progression have also been reported in a range of solid tumor types, including gastric, bladder, and squamous cell carcinoma.[240-242] *PTPN11* mutations are most commonly associated with Noonan syndrome and juvenile myelomonocytic leukemia (JMML), however, activating somatic mutations have also been observed in solid tumors, including colorectal, breast, and renal cell carcinomas.[232,233] These mutations have been shown to enhance cancer progression, invasion, and metastasis,[243-245] and have been associated with decreased

response rates in hepatocellular carcinoma, glioma, and gastric cancers.[243,246,247] In an elegant *in vitro* study utilizing colon and melanoma cancer cells, Prahallad and colleagues demonstrated that *PTPN11* activating mutations were present in the setting of both intrinsic and acquired resistance and that inhibition of *PTPN11* is lethal in cancer cells driven by activated tyrosine kinases.[248]

We identified three genes (*CDK4, CDKN2B,* and *ERBB2*) with differential patterns of copy number alterations between resistant and non-resistant patients. Interestingly, *CDK4* and *CDKN2B* both encode for proteins involved in the cyclin-dependent (cyclin D) pathway, which regulates progression through the cell cycle. The cyclin D pathway is commonly deregulated in solid malignancies through somatic copy number alterations.[249] In our cohort, we observed a higher frequency of *CDKN2B* losses in resistant patients, while non-resistant patients more commonly harbored losses in *CDK4*. Biologically, CDK4 functions as a positive regulator of the cell cycle, while CDKN2B is a negative regulator. Therefore, loss of *CDK4* results in cell cycle arrest and tumor cell senescence, while loss of *CDKN2B* maintains cell cycle progression and tumor cell growth. The CNVs observed in our cohort suggest that cyclin D regulation may serve as an important secondary or bypass track for cancer progression in individuals treated with multi-targeted TKIs. *ERBB2* encodes HER2, a transmembrane tyrosine kinase which regulates the PI3K/AKT pathway upstream of mTOR. Interestingly, after therapeutic failure with multi-targeted TKIs, mTOR inhibitors are recommended as treatment options. We observed that patients non-resistant to multi-targeted TKIs more commonly harbored *ERBB2* losses, which may suggest that mTOR is not overregulated in these individuals, but perhaps is an important mechanism of tumorogenesis in the resistant patients.

Random forest analysis using the combined next-generation sequencing and CNV results

generated a relatively easily translatable decision tree. The genetic alterations that fell out as most informative were copy number variations in *CDKN2B* and *CDK4*, further supporting a potential roll of the cyclin D pathway in resistance to multi-targeted TKIs. Additional genes that fell out as important in decision tree analysis that were not identified in separate next-generation sequencing or CNV analyses were *EPHA3* and *TNFAIP3*, both of which have been previously associated with prognosis and resistance. *EPHA3* encodes a protein tyrosine kinase receptor and its expression has been associated with high invasive capacity and poor overall survival in hepatocellular carcinoma, gastric cancer, and glioblastoma.[250-252] Additionally, *EPHA3* has been associated with the regulation of multi-drug resistance in small cell lung cancer via the PI3K/BMX/STAT3 signaling pathway.[253] *TNFAIP3* encodes a zinc finger protein that serves as a tumor suppressor through its potent inhibition of the NF-κB signaling pathway.[254] *TNFAIP3* has also been associated with regulating drug resistance in multiple solid tumor types.[255,256] Therefore, dysregulation of these genes presents biologically plausible mechanisms of intrinsic resistance to multi-targeted TKIs. While the decision tree generated explained our data well, its ability to describe additional datasets may not be as strong, as demonstrated by leave-one-out cross validation. A larger cohort would improve our ability to more robustly predict intrinsic resistance in independent datasets.

The most notable limitation of this study is the sample size. Due to challenges in procuring patient samples, we were underpowered to detect significant associations after multiple comparisons corrections. With a study of two-to-one non-resistant-to-resistant cases, our data indicate that there is a 5% probability of observing a mutation in the candidate genes. If the true odds ratio for resistance in subjects with mutations relative to non-resistant subjects is 7, we would have needed to study 81 resistant patients and 162 non-resistant patients to be able to

reject the null hypothesis of no difference in odds with a probability (power) equal 80% and a type I error rate equal to 0.0002 (Bonferroni-corrected $p < 0.05$ for 243 genes tested). This highlights the challenge of detecting significant mutations and CNV given the available number of samples. Although we generated whole exome sequencing and whole genome copy number alteration data, we elected to use a candidate gene approach to decrease the multiple testing burden. While this was a good method for statistical purposes, limitations to the candidate gene approach exist, with the main disadvantage being the inability to identify completely novel or unexpected findings. However, the complete paucity of data on resistance to multi-targeted TKIs and, even more-so, intrinsic resistance to multi-targeted TKIs makes any information novel and valuable to this field.

We conducted an exploratory study to identify somatic point mutations and copy number alterations characteristic in individuals with intrinsic resistance to multi-targeted TKIs. We identified potential predictors of resistance that each have some degree of biological plausibility; however, we acknowledge that multiple other factors may be important in determining who will respond to these agents. For example, pharmacokinetics and pharmacodynamics may affect drug penetration and exposure. Using MALDI-MSI (matrix-assisted laser desorption ionization mass spectrometry imaging) to visualize the distribution patterns of multi-targeted TKIs in mouse models, Torok and colleagues determined that poor drug penetration in some tumors resulted in primary resistance.[257] Clinical variability in drug concentrations, despite receiving the same dose, has also been observed and associated with variability in side effects and survival outcomes.[258,259] Lysosomal sequestration has also been demonstrated to confer resistance to the multi-targeted TKIs[148,149] and has also been associated with conferring cross-resistance to the multi-targeted TKIs.[142] Finally, germline genetics in drug transporters, such as *ABCB1* and

ABCG2, or pharmacodynamic proteins, such as *BIM*, may also influence response to these agents.[10,29,260] While we identified somatic markers of resistance to multi-targeted TKIs, there are many factors that must be considered when developing an optimized predictive algorithm for predicting resistance in clinical practice.

Tables and Figures

Table 5.1: Patient demographics (n = 49). Demographics are also broken down by phenotype for individuals who underwent next-generation sequencing (n=37) and copy number variation analysis (n=29).

		Next-generation sequencing			Copy number variation		
Characteristic	**All patients (n = 50)**	**Resistant (n = 11)**	**Non-resistant (n = 26)**	**P-value (FDR)[#]**	**Resistant (n = 8)**	**Non-resistant (n = 21)**	**P-value (FDR)[#]**
Age*				0.49 (0.55)			0.25 (0.31)
Mean ± SD	59.3 ± 12	57.4 ± 12.7	60.6 ± 12.1		58.8 ± 13.9	64.1 ± 10.0	
Median	61	56	61.5		60	67	
Range	36 – 81	37 – 79	36 – 81		37 – 79	39 – 81	
Sex				0.05 (0.15)			0.03 (0.07)
Male	36 (72)	7 (63.6)	24 (92.3)		4 (50)	19 (90.5)	
Female	14 (28)	4 (36.3)	2 (7.7)		4 (50)	2 (9.5)	
Race				0.16 (0.24)			0.07 (0.10)
White	43 (86)	9 (81.8)	24 (92.3)		6 (75)	21 (100)	
Black	4 (8)	2 (18.2)	0		2 (25)	0	
Asian	2 (4)	0	1 (3.8)		0	0	
Unknown	1 (2)	0	1 (3.8)		0	0	
Ethnicity				0.65 (0.65)			1.0 (1.0)
Hispanic	7 (14)	1 (9.1)	5 (19.2)		1 (12.5)	3 (14.3)	
Non-Hispanic	43 (86)	10 (90.9)	21 (80.8)		7 (87.5)	18 (85.7)	
Cancer type				0.08 (0.16)			0.02 (0.07)
Sarcoma	26 (52)	6 (54.5)	12 (46.1)		4 (50)	7 (33.3)	
Renal cell carcinoma	18 (36)	2 (18.2)	13 (50)		1 (12.5)	13 (61.9)	
Hepatic	4 (8)	2 (18.2)	1 (3.8)		2 (25)	1 (4.8)	
Colorectal	1 (2)	1 (9.1)	0		1 (12.5)	0	
Melanoma	1 (2)	0	0		0	0	
Multi-targeted TKI				0.03 (0.15)			0.007 (0.04)
Pazopanib	21 (42)	7 (63.6)	6 (23.1)		5 (62.5)	3 (14.3)	
Sorafenib	15 (30)	2 (18.2)	11 (42.3)		2 (25)	10 (47.6)	
Sunitinib	13 (26)	1 (9.1)	9 (34.6)		0	8 (38.1)	
Regorafenib	1 (2)	1 (9.1)	0		1 (12.5)	0	

*Age represents the age at multi-targeted TKI initiation.
[#]P-value for continuous variables represents the Mann-Whitney U p-value and categorical data was compared between resistant and non-resistant individuals using Fisher's exact test. FDR represents the FDR-corrected p-value.

Figure 5.1: Time to multi-targeted tyrosine kinase inhibitor by phenotype. Data represents mean and standard deviation. **Mann-Whitney U p-value < 0.0001

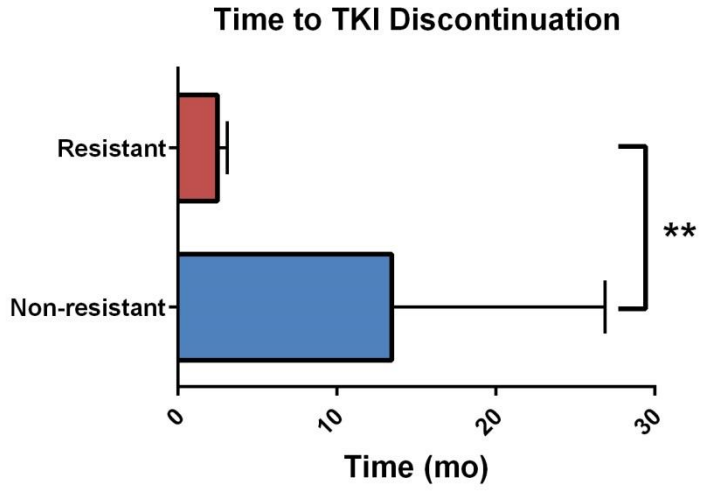


Figure 5.2: OncoPrint of somatic gene mutations observed differentially by phenotype.

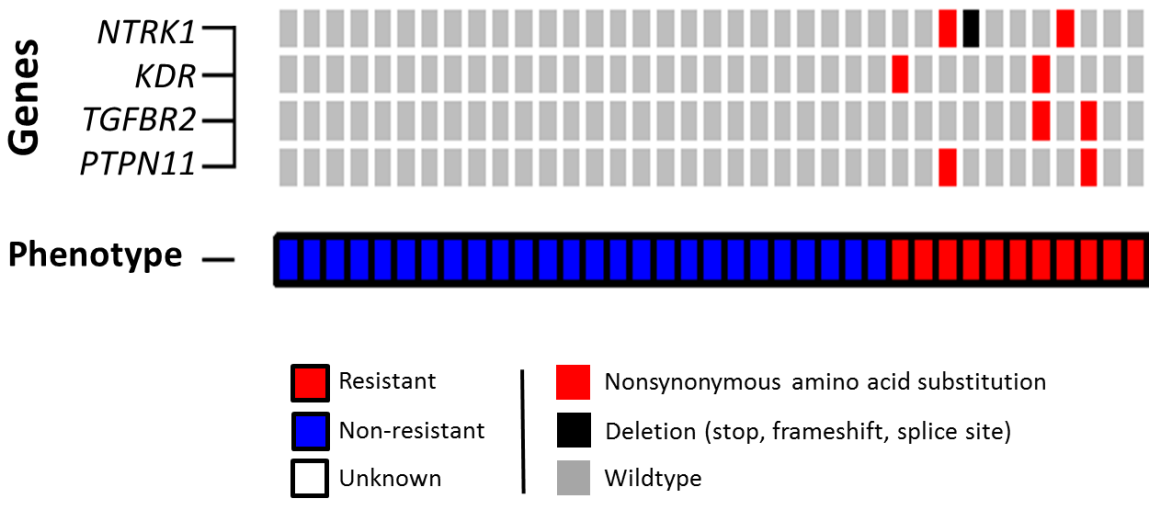


Figure 5.3: Percent of patients with somatic nonsynonymous point mutations or splice site variants in the four top gene hits.

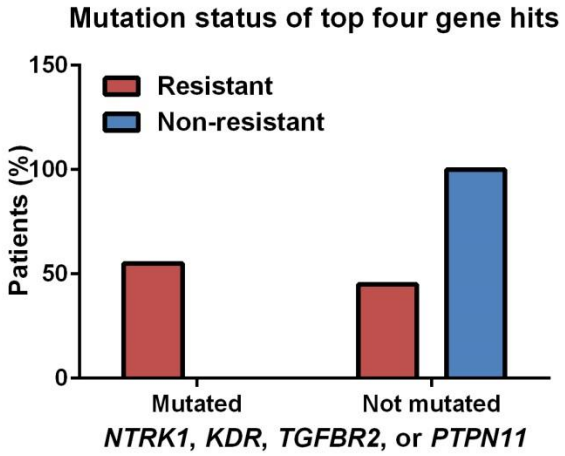


Figure 5.4: Copy number variations (CNVs) observed by phenotype. *Genes that met pre-specified cut-off for exploratory hits (i.e., differential CNVs between resistant and non-resistant individuals).

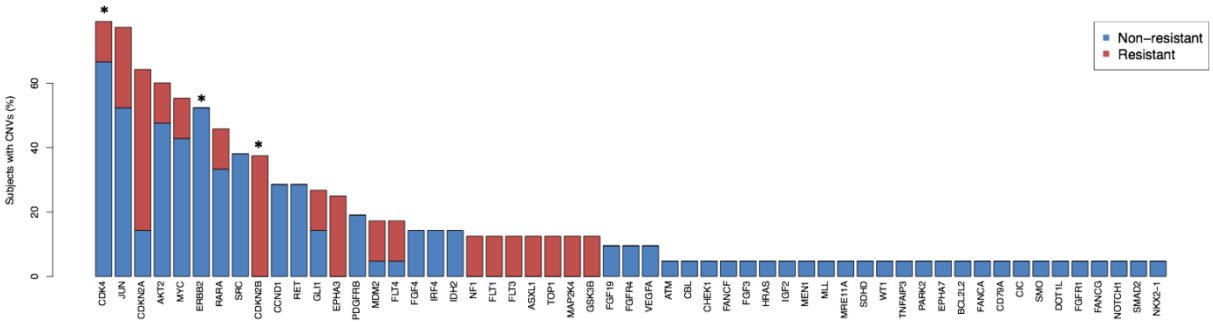
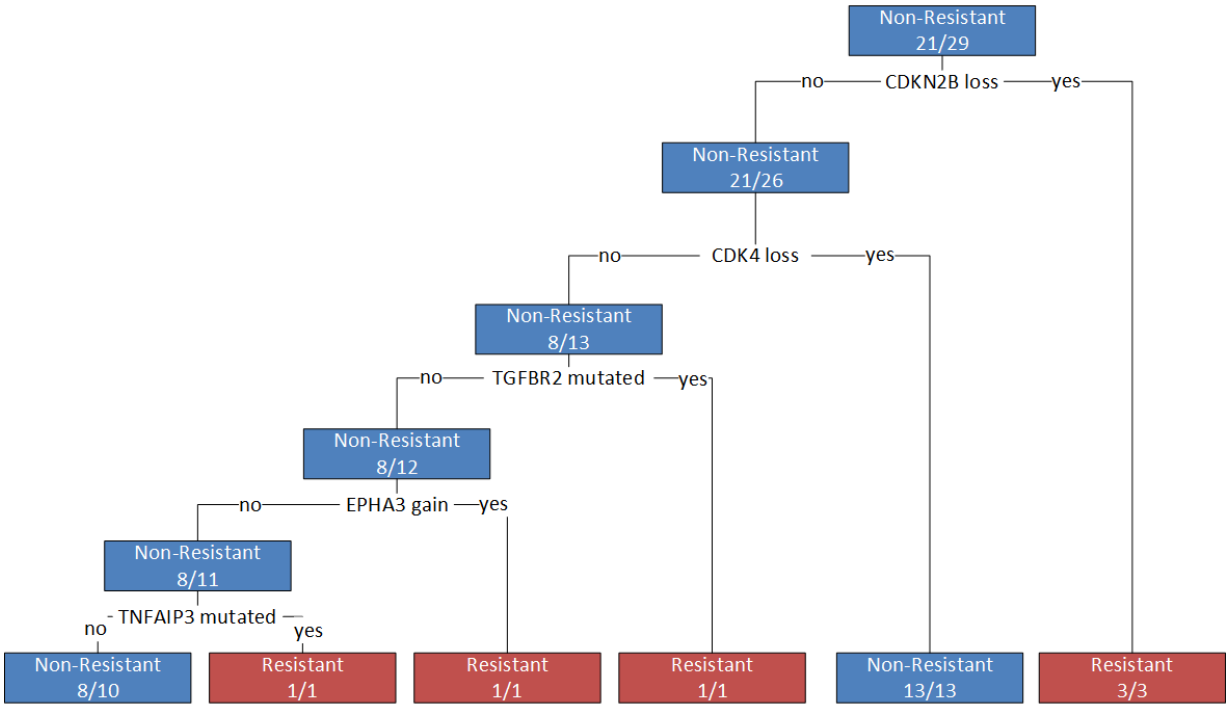


Figure 5.5: Decision tree for differentiating resistant from non-resistant patients. Branches were generated from genes that were identified as influential in differentiating phenotypes using a random forest classification model. A loss represents a homozygous copy number loss; a gain represents a copy number gain greater than seven; a mutation represents any non-synonymous or missense mutation in a coding region of the gene.



CHAPTER 6: SIGNIFICANCE AND FUTURE DIRECTIONS

Current challenges in the personalization of multi-targeted TKI prescribing

This dissertation presents the first attempt at understanding the clinical and genetic factors mediating intrinsic resistance and detail the severe cutaneous toxicity risk to the widely prescribed multi-targeted TKIs. As discussed in Chapter 1, extensive data exists demonstrating that tumors acquire genetic alterations that confer secondary, or acquired, resistance to protein kinase inhibitors after an initial response period;[17,80] however, there is a paucity of data describing mechanisms of intrinsic resistance. Specifically, there are no data available describing mechanisms of intrinsic resistance to the multi-targeted TKIs, despite up to thirty percent non-response rates across tumor types. Through the aims of this dissertation, we provide the first data demonstrating potential clinical and genetic factors that may be important for optimizing the prescribing of the multi-targeted TKIs.

One of the most common reasons for inefficacy of anticancer agents is drug discontinuation or dose reductions due to side effects. In Chapter 2, we conducted the first large-scale study of the incidence and triggers of the extremely rare life-threatening adverse events, Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN), which have been linked to tyrosine kinase inhibitors in multiple case reports.[176,178,180] We screened 108,825 cancer patients and identified 121 with diagnosis codes for SJS/TEN. Using this large cohort, we were able to, for the first time, confirm a higher incidence rate of SJS/TEN in cancer patients than in the general population. Almost 5,000 individuals screened had received treatment with a TKI and we observed a possible prevalence of up to 60 cases per 100,000 individuals in this

subgroup, suggestive of an elevated risk; however, the incidence was too low to definitively confirm a link between TKI administration and SJS/TEN. The primary limitations of this study are inherent to the diagnosis and treatment of SJS/TEN, and include the lack of definitive diagnostic criteria and the severity of the reaction prohibiting definitive confirmation of presumed triggers. Our results, coupled with the increased mortality rate from SJS/TEN observed in cancer patients,[183] emphasize the importance of early detection of SJS/TEN in cancer patients and warrant additional studies to confirm anticancer agents that may trigger this life-threatening event.

The retrospective study conducted in Chapter 3 provides insight into the demographic and clinical factors that may be important to incorporate into a predictive algorithm of intrinsic resistance to the multi-targeted TKIs. Concordance in classifying patients as intrinsically resistant or non-resistant by independent reviewers and the observed intrinsic rate falling in line with that reported in clinical trials and the literature[136,141,185] provided support for the phenotyping methodology utilized for this and other aims. Failure to observe associations between resistance and demographics, tumor types, or multi-targeted TKIs received provides important evidence to support our original hypothesis that prescribing multi-targeted TKIs solely based on tumor type and FDA-approved indications neglects other factors important in optimizing individualized patient outcomes. The finding of similar rates of intrinsic resistance in various demographic groups, tumor types, and with multiple multi-targeted TKIs provides evidence there may be other unidentified shared markers, perhaps genetic, of resistance to these agents across tumor types.

To our knowledge, Chapter 4 is the first methodology study exploring the utility of FFPE-derived DNA from a range of ages and tissue types as a source for next-generation

sequencing of tumor samples. To our surprise, we found that age of the FFPE block does not determine the quality of next-generation sequencing results, but rather tissue source may influence DNA yield and affect the ability to generate usable next-generation sequencing data. Specifically, we found that FFPE samples derived from bone tissue tended to have low or insufficient DNA yield. This study provides invaluable insight into the utility of the over one billion FFPE blocks stored in hospitals around the world,[187] confirming their potential as resources for retrospective studies. Furthermore, this chapter provided the methodology for executing our genetic analysis aim and cautioned against the utility of bone-derived FFPE samples with these methods.

Chapter 5 discusses the first study exploring somatic genetics as a predictor of intrinsic resistance to multi-targeted TKIs and presents data supporting an association between somatic mutations and copy number alterations and resistance. The hypothesis of genetics affecting response to multi-targeted TKIs was supported by the results of Chapter 3 and our methods for this aim were validated in Chapter 4. Also included in Chapter 5 is an easily translatable decision tree model that incorporates point mutations and copy number variations to discriminate between resistant and non-resistant patients. We observed a thirty percent resistance rate in our study cohort, consistent with that previously reported.[141] The genes that fell out in each component of the genetic analysis represented biologically plausible predictors of resistance that have been previously associated with resistance to other anticancer agents.[229,243,256] Some of our gene hits were also predicted to confer resistance to multi-targeted TKIs using *in vivo* models.[227,228] The most interesting and most informative gene hits identified in these analyses, *CDK4* and *CDKN2B*, harbored opposite frequencies of copy number losses between resistant and non-resistant patients, which is intriguing because the genes encode for opposing regulators of the

same pathogenic pathway (i.e., one negatively regulates and one positively regulates the cyclin D pathway). The results presented in Chapter 5 provide a strong foundation for future studies aimed at confirming mechanisms of intrinsic resistance to multi-targeted TKIs.

The primary limitation of the aim addressed in Chapter 5 was the sample size. While we were able to identify genetic associations at pre-defined exploratory cut-offs in the largest study conducted to date, none of the gene hits met statistical significance after applying an FDR correction for multiple comparisons; however, this is commonly the case for genetic studies in cancer, where most studies of resistance include data from less than one hundred patients. For example, the study that identified *TSC1* as predictive of everolimus response consisted of 13 tumor samples and many other high-impact publications are single-patient case reports.[127,210,261] In Chapter 5 we provided estimates for the number of samples that would be needed to meet statistical significance given our results. The sample size also limited our ability to conduct stratified analyses of our cohort, although our Chapter 3 results suggest that stratification may not be necessary. Unlike most studies, the major challenge in including sufficient individuals for this study was not in the identification of eligible individuals, the recruitment process, or sample availability, but was in tissue procurement. Nonetheless, we identified a series of strong candidate genes that warrant further investigation in the setting of intrinsic resistance to multi-targeted TKIs.

Implications for current research and clinical practice

While the results generated in this dissertation are not sufficient to warrant immediate clinical implementation, they provide great insight into the previously underexplored phenomenon of intrinsic resistance to targeted anticancer agents. Our results contribute to the existing literature[205,206] supporting shared molecular mechanisms of oncogenicity across various

histological tumor types, as discussed in Chapters 1 and 5, and provide novel evidence that there are likely shared mechanisms of resistance within the class of multi-targeted TKIs. Taken together, our data suggest that histological tumor type is unlikely the only crucial factor to determine a patient's indication for multi-targeted TKIs and that genetics may also be important to consider before prescribing these agents. Coupled with the significant rates of intrinsic resistance to multi-targeted TKIs observed in clinical studies and in practice, our results further emphasize the limitation of histological-based prescribing that is the current standard practice for the vast majority of solid tumor types.

The data presented in this dissertation also provide insight into future research avenues. First, our results suggest that copy number alterations may be a more important determinate of resistance to multi-targeted TKIs than point mutations, a novel insight that may help prioritize future validation studies of this work. The methodological validation of next-generation sequencing using diverse FFPE samples provides evidence to support the broad use of FFPE samples for research. Since FFPE blocks are the standard method for clinical tissue storage and are generally linked with detailed medical records, the availability of these samples for genetic analyses provides an invaluable resource for retrospective studies. Specifically, in Chapter 4 we demonstrate that FFPE samples from a range of ages are potentially useful, therefore broadening the utility and potential impact of this resource.

The confirmation of a heightened incidence of SJS and TEN in cancer patients, described in Chapter 2, directly impacts clinical practice. These results along with the life-threatening nature of SJS/TEN, highlight the importance of educating oncologists on early identification and management of SJS/TEN. The link we observed between some cases and anticancer agents emphasizes the necessity of additional studies exploring the triggers of SJS/TEN and the

pathological mechanism of the reaction. Novel methods are needed to definitively identify triggers in a high-throughput manner. Additionally, the mechanism of increased risk in certain diseases, such as cancer or HIV,[167,181] and elucidation of genetic factors (e.g., HLA status)[182] warrant additional investigation. Ultimately, a thorough understanding of the risk factors for SJS/TEN will allow development of a comprehensive algorithm to determine highest risk patients and guide clinical decisions.

Opportunities for personalized multi-targeted TKI prescribing

This dissertation provides the foundation for multiple opportunities to optimize personalized prescribing of multi-targeted TKIs. First, if we could pre-emptively identify patients at risk for SJS/TEN, we could increase monitoring or withhold TKI treatment altogether. A comprehensive assessment of additional clinical factors, including diseases, drug exposures, genetics, and potentially immune system status would greatly improve our current understanding of why some individuals experience SJS/TEN when exposed to commonly prescribed medications. Due to the extremely rare incidence of this reaction, collaborative studies are needed to generate sufficient evidence for the development of a translatable decision algorithm to help inform personalized prescribing and monitoring of potential triggers, including multi-targeted TKIs.

The finding that long-time archived FFPE blocks are sufficient for next-generation sequencing can be implemented in clinical practice and research. For example, in the absence of fresh frozen samples, archived samples may be informative in understanding mechanisms of resistance in an individual's tumor. If a patient was once responding to a targeted agent, but later stopped responding, their archived tumor sample may be helpful in understanding molecular changes in the tumor over time that mediated resistance. Likewise, if a patient fails to respond to

a treatment from time of initiation, analysis of their archived tumor block may be informative in understanding the mechanism of intrinsic resistance (if mediated by somatic genetics). This type of information would be beneficial to both the patient and the field of oncology, further elucidating potential mechanisms of resistance to targeted therapies.

Future studies are warranted to confirm the genetic associations with intrinsic resistance reported in this dissertation before being implemented clinically. Additional studies exploring other potentially relevant mechanisms of intrinsic resistance, such as drug exposure, drug penetration, and germline pharmacogenetics may help develop a more refined prediction algorithm of intrinsic resistance to multi-targeted TKIs. Once validated in additional studies, implementation of these results has the potential to impact the many thousands of cancer patients treated with multi-targeted TKIs. Incorporation of genetic and clinical factors into an easily translatable decision algorithm will save physicians and patients valuable time, decrease the risk of unnecessary exposure to dangerous side effects, and save money.

Conclusion

This dissertation describes a series of innovative studies conducted to better understand the clinically significant problem of intrinsic resistance to multi-targeted TKIs. We conducted a series of retrospective studies to explore demographic, clinical, and genetic factors that may predict resistance to these agents. This work provides the first steps toward creating a personalized decision algorithm for multi-targeted TKI prescribing, which has the potential to greatly improve outcomes for up to thirty percent of patients with histologically-based indications for these agents.

APPENDIX 2.1: STEVENS-JOHNSON SYNDROME OR TOXIC EPIDERMAL NECROLYSIS CULPRIT AGENTS AS NOTED BY PHYSICIANS

Classification	Trigger	Trigger (Figure 2.1)
Confirmed	sulfamethoxazole/trimethoprim, foscarnet, bumetanide, furosemide, levofloxacin, cefepime, "amongst other things"	ANTIBIOTIC, ANTIVIRAL
Confirmed	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Confirmed	cefepime	ANTIBIOTIC
Confirmed	sulfamethoxazole/trimethoprim and/or probenecid	ANTIBIOTIC, ANTI-GOUT
Confirmed	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Confirmed	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Confirmed	phenytoin vs. sulfonamides	ANTIBIOTIC, ANTICONVULSANT
Confirmed	cefepime vs. vancomycin	ANTIBIOTIC
Confirmed	phenytoin	ANTICONVULSANT
Confirmed	aztreonam and penicillin	ANTIBIOTIC
Confirmed	vancomycin or cefepime	ANTIBIOTIC
Confirmed	piperacillin/tazobactam	ANTIBIOTIC
Confirmed	sulfamethoxazole/trimethoprim vs. methotrexate	ANTIBIOTIC, IMMUNOMODULATOR
Confirmed	piperacillin/tazobactam	ANTIBIOTIC
Confirmed	irinotecan vs. phenytoin	ANTINEOPLASTIC,

		ANTICONVULSANT
Confirmed	chemo (etoposide, methotrexate, dactinomycin, cisplatin, filgrastim, leucovorin) vs. piperacillin/tazobactam	ANTIBIOTIC, ANTINEOPLASTIC, IMMUNOMODULATOR
Confirmed	allopurinol	ANTI-GOUT
Confirmed	cefepime, piperacillin/tazobactam, aztreonam, meropenem, vancomycin, tobramycin, voriconazole	ANTIBIOTIC, ANTIFUNGAL
Confirmed	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Confirmed	lenalidomide	IMMUNOMODULATOR
Possible	phenytoin	ANTICONVULSANT
Possible	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Possible	interferon	IMMUNOMODULATOR
Possible	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Possible	MLN0264 (antibody-drug conjugate targeting guanylyl cyclase C), ceftriaxone, amoxicillin/clavulanate, piperacillin/tazobactam	ANTIBIOTIC, MONOCLONAL ANTIBODY
Possible	sulfa-	ANTIBIOTIC
Possible	capecitabine	ANTINEOPLASTIC
Possible	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Possible	Herpes simplex virus vs. cyclophosphamide vs. doxorubicin	ANTINEOPLASTIC, VIRUS

Possible	sunitinb or sorafenib	ANTINEOPLASTIC
Possible	apitolisib vs. paclitaxel	ANTINEOPLASTIC
Possible	Merck PD1 antibody (pembrolizumab?)	MONOCLONAL ANTIBODY
Possible	Herpes simplex virus vs. allopurinol, levofloxacin, or famciclovir	ANTIBIOTIC, ANTI-GOUT, ANTIVIRAL
Possible	Herpes simplex virus	VIRUS
Possible	bisphosphonate	MISC.
Possible	levofloxacin	ANTIBIOTIC
Possible	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Possible	caspofungin vs. vancomycin	ANTIBIOTIC, ANTIFUNGAL
Possible	temozolomide	ANTINEOPLASTIC
Possible	phenytoin	ANTICONVULSANT
Possible	epoetin alfa	MISC.
Possible	colchicine	ANTI-GOUT
Possible	unknown	UNKNOWN
Possible	unknown	UNKNOWN
Possible	phenytoin	ANTICONVULSANT
Possible	minocycline	ANTIBIOTIC
Possible	likely sulfa	ANTIBIOTIC
Possible	saline	MISC.
Possible	fluconazole	ANTIFUNGAL

Possible	unknown	UNKNOWN
Historical	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Historical	unknown	UNKNOWN
Historical	estrogen or ibuprofen	MISC.
Historical	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Historical	penicillin	ANTIBIOTIC
Historical	erythromycin	ANTIBIOTIC
Historical	voriconazole	ANTIFUNGAL
Historical	sulfamethoxazole/trimethoprim	ANTIBIOTIC
Historical	unknown	UNKNOWN
Historical	clindamycin	ANTIBIOTIC
Historical	unknown	UNKNOWN
Historical	clobetasol	MISC.

APPENDIX 3.1: SOURCE DATA FOR DISCRETE VARIABLES ABSTRACTED FROM THE TOTAL CANCER CARE BIOREPOSITORY

Abbreviations – EMR: electronic medical record, TKI: tyrosine kinase inhibitor

Data Variable	Data Source
Age at diagnosis	Derived from date of diagnosis (Cancer Registry) minus date of birth (EMR)
Sex	Cancer Registry
Race	Cancer Registry
Ethnicity	Cancer Registry and EMR
Primary Site	Cancer Registry
Histology	Cancer Registry
First course of treatment	Cancer Registry
First course of treatment facility	Cancer Registry
Multi-targeted TKI received	Cancer Registry and EMR
Start and stop date of TKI	EMR

APPENDIX 4.1: BREAKDOWN OF FFPE SAMPLES FOR DNA EXTRACTION AND WHOLE EXOME SEQUENCING

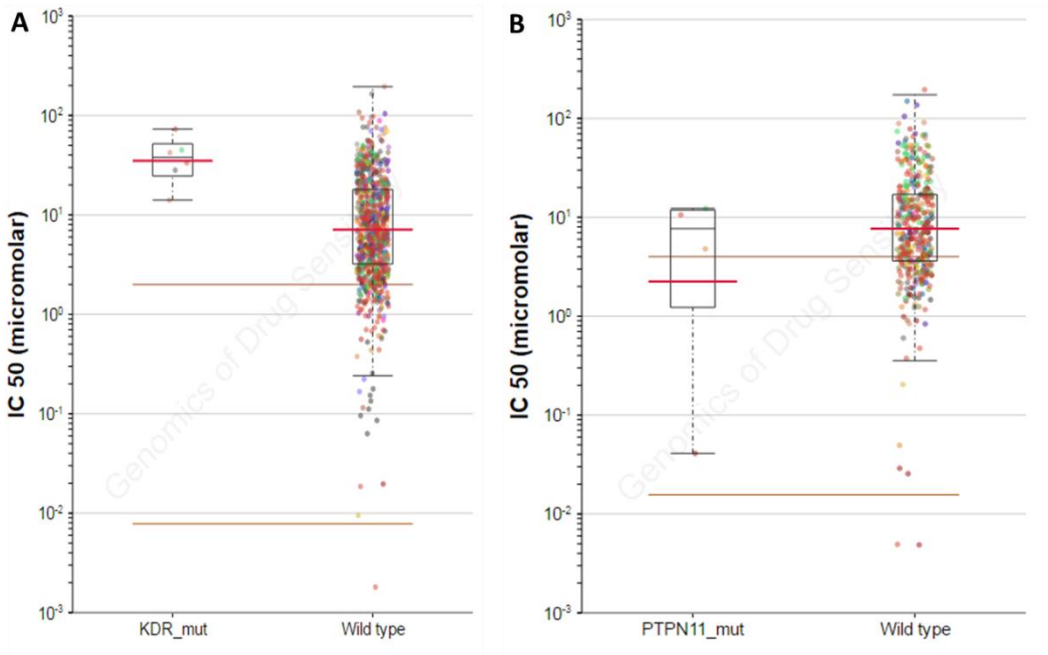
Sample age (yr)	GOAL		IDENTIFIED		RECEIVED	
	RCC (n)	Sarcoma (n)	RCC (n)	Sarcoma (n)	RCC (n)	Sarcoma (n)
1-3	3	2	6	4	4	4
3-5	3	2	3	7	1	5
5-7	3	2	3	6	1	5
7-10	3	2	3	4	0	4
>10	3	2	3	4	0	4
TOTAL	15	10	18	25	6	22
	25		**43**		**28**	

APPENDIX 5.1: CANDIDATE GENES INCLUDED IN NEXT-GENERATION SEQUENCING AND COPY NUMBER VARIATION ANALYSES

ABL1	ABL2	ACVR1B	AKT1	AKT2	AKT3	ALK	APC
AR	ARAF	ARFRP1	ARID1A	ASXL1	ATM	ATR	ATRX
AURKA	AURKB	AXIN1	AXL	BAP1	BARD1	BCL2	BCL2L1
BCL2L2	BCL6	BCORL1	BLM	BRAF	BRCA1	BRCA2	BRIP1
BTK	CARD11	CBL	CCND1	CCND2	CCND3	CCNE1	CD79A
CD79B	CDC73	CDH1	CDK12	CDK4	CDK6	CDK8	CDKN1A
CDKN1B	CDKN2A	CDKN2B	CDKN2C	CEBPA	CHEK1	CHEK2	CIC
CREBBP	CRKL	CRLF2	CSF1R	CTNNA1	CTNNB1	CYLD	DAXX
DDR2	DNMT3A	DOT1L	EGFR	EP300	EPHA3	EPHA5	EPHA7
EPHB1	ERBB2	ERBB3	ERBB4	ERG	ERRFI1	ESR1	EZH2
FANCA	FANCC	FANCD2	FANCE	FANCF	FANCG	FANCL	FAS
FBXW7	FGF10	FGF19	FGF3	FGF4	FGFR1	FGFR2	FGFR3
FGFR4	FH	FLT1	FLT3	FLT4	FOXL2	GATA1	GLI1
GNA11	GNAQ	GNAS	GRIN2A	GRM3	GSK3B	HGF	HNF1A
HRAS	HSP90	IDH1	IDH2	IGF1R	IGF2	IKBKE	IKZF1
INHBA	INPP4B	IRF4	IRS2	JAK1	JAK2	JAK3	JUN
KDM5A	KDM5C	KDM6A	KDR	KEAP1	KIT	KRAS	LRP1B
LYN	MAGI2	MAP2K1	MAP2K2	MAP2K4	MAP3K1	MCL1	MDM2
MDM4	MEN1	MET	MITF	MLH1	MLL	MLL2	MLL3
MPL	MRE11A	MSH2	MSH6	MTOR	MUTYH	MYC	MYCN
MYD88	NF1	NF2	NKX2-1	NOTCH1	NOTCH2	NOTCH3	NPM1
NRAS	NTRK1	NTRK2	NTRK3	PAK3	PALB2	PARK2	PAX5
PDGFRA	PDGFRB	PDK1	PIK3C2B	PIK3CA	PIK3CB	PIK3CG	PIK3R1
PIK3R2	PLCG2	PMS2	POLE	PPP2R1A	PREX2	PRKAR1A	PRKCI
PRKDC	PTCH1	PTEN	PTPN11	RAC1	RAD50	RAD51	RAF1
RARA	RB1	RET	RICTOR	ROS1	RPTOR	RUNX1	RUNX1T1
SDHB	SDHC	SDHD	SETD2	SMAD2	SMAD3	SMAD4	SMARCA4
SMARCB1	SMO	SOCS1	SOX10	SOX2	SPEN	SPOP	SRC
STAT3	STAT4	STK11	SUFU	SYK	TERT	TET2	TGFBR2
TNFAIP3	TOP1	TOP2A	TP53	TSC1	TSC2	TSHR	VEGFA
VHL	WT1	ZNF217					

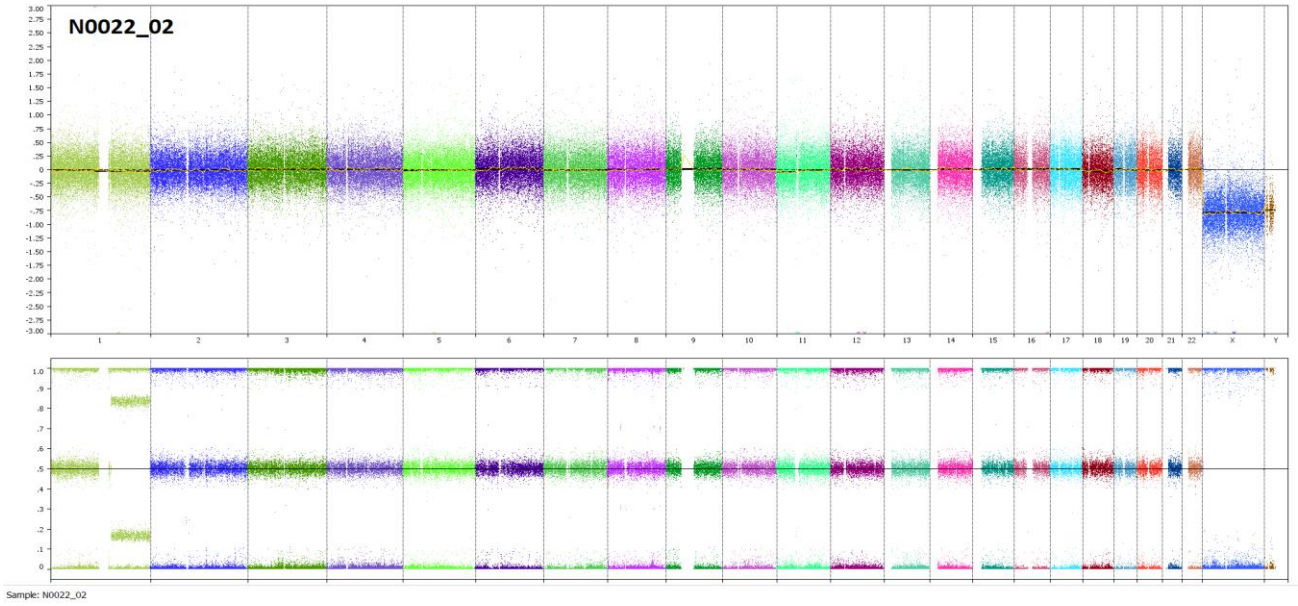
APPENDIX 5.2: EXAMPLE GRAPH GENERATED USING THE GENOMICS OF DRUG SENSITIVITY IN CANCER DATABASE[221,227]

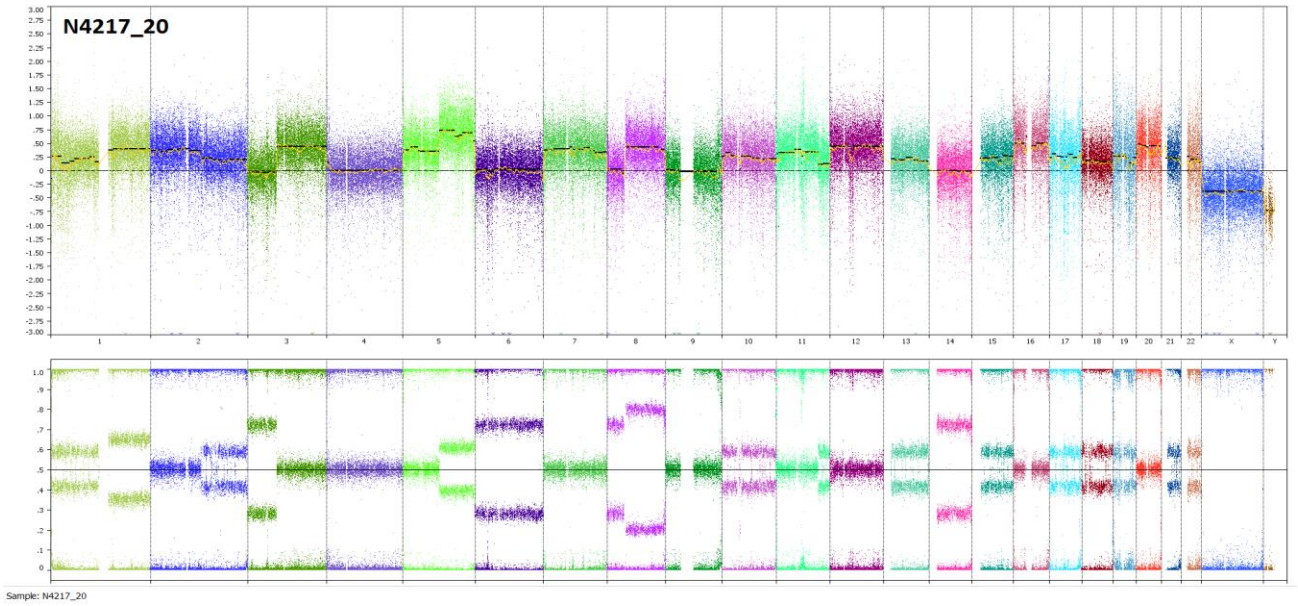
(A) Represents the effect of *KDR* mutations on the IC_{50} value for axitinib in cancer cell lines screened (represented by different color dots). In this example, *KDR* mutations increase the IC_{50}, suggesting resistance to axitinib. (B) Represents the effect of *PTPN11* mutations on the IC_{50} for sorafenib in cancer cell lines. In this example, *PTPN11* mutations decrease the IC_{50}, suggesting potential increased sensitivity to sorafenib.



APPENDIX 5.3: WHOLE-GENOME COPY NUMBER VARIATION PLOTS

Plots represent data from an individual with relatively stable genomic copy number (top two graphs) and an individual with instable whole genome copy number aberrations (bottom two graphs). The top graphs for each individual display whole genome copy number alterations, where > 0 represents a gain and < 0 represents a loss. The bottom graphs for each individual present B allele frequency information, which enables detection of mosaic gains and losses and loss of heterozygosity.





APPENDIX 5.4: MEAN DECREASE GINI SCORES FOR DECISION TREE ANALYSIS

Values for non-resistant and resistant represent the mean raw importance score for the alteration for classification of that genotype. The mean decrease accuracy represents the proportion of observations that are incorrectly classified by removing the alteration. The mean decrease Gini score represents the number of splits (across all trees) that include that alteration, proportionally to the number of samples it splits. Therefore, the larger the decrease Gini score, the more important that alteration is in differentiating resistant from non-resistant individuals. Alterations with a mean decrease Gini > 0.2 were included in the decision tree (highlighted yellow). Bolded alterations are those that fell out as important factors in the final decision tree model. Remaining factors with a mean decrease Gini > 0.2 that are not in the decision tree are still important, but do not add any additional information to those selected for inclusion in the model.

	Non-resistant	Resistant	Mean Decrease Accuracy	Mean Decrease Gini Score
cnv_CDKN2B	**0.0106**	**0.0255**	**0.0145**	**0.6324**
wes_TGFBR2	**0.0040**	**0.0073**	**0.0045**	**0.3909**
cnv_EPHA3	**0.0039**	**0.0070**	**0.0047**	**0.3764**
cnv_CDK4	**0.0004**	**0.0293**	**0.0070**	**0.3686**
cnv_ERBB2	**0.0038**	**0.0264**	**0.0084**	**0.3444**
wes_KDR	0.0025	0.0054	0.0032	0.3405
wes_PTPN11	0.0039	0.0067	0.0045	0.3310
cnv_CDKN2A	0.0014	0.0065	0.0026	0.3009
wes_TNFAIP3	**0**	**0**	**0**	**0.2865**
wes_NTRK1	0.0024	0.0045	0.0029	0.2431
wes_RICTOR	-0.0077	-0.0094	-0.0074	0.2257
wes_TERT	0.0010	0.0016	0.0011	0.2182
wes_SDHD	0	0	0	0.1691
wes_PRKDC	-0.0006	-0.0022	-0.0009	0.1689
wes_IKZF1	0.0021	-0.0028	0.0006	0.1681
cnv_SRC	-0.0007	0.0094	0.0017	0.1604
wes_APC	-0.0014	-0.0022	-0.0016	0.1521
wes_AR	-0.0013	-0.0062	-0.0022	0.1493
cnv_JUN	-0.0041	0.0060	-0.0016	0.1411

cnv_AKT2	-8.91E-06	0.0071	0.0016	0.1369
wes_IKBKE	-0.0011	-0.0023	-0.0014	0.1345
wes_VHL	0.0004	0.0070	0.0018	0.1324
cnv_MYC	-0.0034	0.0029	-0.0019	0.1234
cnv_TOP1	0	0	0	0.1233
wes_NOTCH2	-0.0007	-0.0032	-0.0015	0.1229
wes_JAK2	0.0024	0.0049	0.0030	0.1156
wes_AXIN1	-0.0011	-0.0026	-0.0014	0.1120
cnv_FLT1	0	0	0	0.1120
wes_NRAS	0	0	0	0.1119
cnv_FLT3	0	0	0	0.1100
cnv_GSK3B	0	0	0	0.1091
cnv_NF1	0	0	0	0.1089
cnv_ASXL1	0	0	0	0.1053
cnv_MAP2K4	0	0	0	0.1048
wes_CBL	0	0	0	0.1047
wes_JAK3	-0.0005	-0.0038	-0.0013	0.1035
wes_NOTCH1	0	0	0	0.1032
wes_FOXL2	-0.0002	0.0005	-0.0001	0.0969
wes_ATR	-0.0004	0.0039	0.0006	0.0949
cnv_CCND1	-0.0013	0.0056	0.0001	0.0941
wes_PMS2	-0.0014	-0.0019	-0.0014	0.0941
wes_MLL	-0.0007	-0.0011	-0.0008	0.0937
wes_DAXX	0	0	0	0.0918
wes_STAT4	0	0	0	0.0882
wes_PIK3C2B	0	0	0	0.0878
wes_FLT4	-0.0005	-0.0031	-0.0010	0.0850
wes_BRCA2	-7.72E-05	-0.0024	-0.0006	0.0843
wes_MLL3	-0.0022	0.0015	-0.0013	0.0829
wes_BARD1	0.0002	-0.0009	-4.66E-05	0.0820
wes_ATRX	0	0	0	0.0813
wes_TP53	-2.52E-05	-0.0023	-0.0006	0.0744
cnv_RARA	-0.0019	0.0010	-0.0010	0.0713
cnv_RET	-0.0010	0.0041	9.75E-06	0.0667
wes_FLT1	-0.0006	-0.0014	-0.0008	0.0665
wes_MDM2	-0.0006	-0.0007	-0.0007	0.0566
wes_BAP1	0.0005	0.0008	0.0006	0.0561
cnv_FLT4	-0.0004	-0.0007	-0.0005	0.0545
wes_POLE	0.0005	0.0013	0.0006	0.0544
wes_EPHA5	-0.0002	0.0008	1.13E-05	0.0540

wes_CDH1	-0.0003	-0.0007	-0.0003	0.0508
cnv_MDM2	-0.0004	-0.0005	-0.0004	0.0483
wes_CIC	-0.0008	-0.0021	-0.0010	0.0472
wes_SETD2	-0.0012	-8.33E-05	-0.0009	0.0464
wes_PTCH1	0	0	0	0.0461
wes_KIT	0	0	0	0.0411
wes_PDGFRB	0	0	0	0.0410
wes_MLH1	0	0	0	0.0384
wes_PREX2	-0.0002	0.0001	-0.0001	0.0355
wes_TSC1	-0.0002	0.0004	-5.32E-05	0.0345
wes_KDM5C	-0.0001	0.0010	0.0001	0.0344
wes_SDHC	0	0	0	0.0300
wes_JUN	0	0	0	0.0296
wes_KDM5A	-0.0006	-0.0005	-0.0005	0.0296
cnv_GLI1	-0.0006	-0.0016	-0.0007	0.0286
wes_CDKN1B	0	0	0	0.0268
wes_RAF1	0	0	0	0.0257
wes_SMO	0	0	0	0.0253
wes_HNF1A	0	0	0	0.0232
wes_TOP2A	0	0	0	0.0218
wes_NTRK2	0	0	0	0.0218
wes_FANCL	0	0	0	0.0215
cnv_PARK2	0	0	0	0.0207
cnv_PDGFRB	-0.0004	0.0011	-8.47E-05	0.0202
wes_ROS1	0	0	0	0.0198
wes_FGFR3	0	0	0	0.0198
cnv_EPHA7	0	0	0	0.0198
wes_ALK	0	0	0	0.0195
cnv_FGF4	-6.81E-05	0.0005	8.26E-05	0.0190
wes_EGFR	0	0	0	0.0182
cnv_TNFAIP3	0	0	0	0.0176
wes_AKT1	0	0	0	0.0174
wes_BCL6	0	0	0	0.0161
wes_BCORL1	-0.0002	0.0009	-1.41E-05	0.0158
cnv_IDH2	-8.51E-05	0.0003	2.59E-05	0.0158
wes_FH	-0.0005	0.0001	-0.0003	0.0142
wes_FANCG	0	0	0	0.0141
wes_FANCA	0	0	0	0.0137
cnv_FGF19	0	0	0	0.0136

wes_PDGFRA	0	0	0	0.0136
cnv_IGF2	0	0	0	0.0133
cnv_IRF4	-8.33E-05	0.0005	-2.40E-05	0.0130
cnv_WT1	0	0	0	0.0122
wes_BRIP1	0	0	0	0.0120
wes_ERBB3	0	0	0	0.0117
cnv_VEGFA	-6.25E-05	0	-5.00E-05	0.0099
cnv_ATM	0	0	0	0.0098
cnv_MRE11A	0	0	0	0.0093
wes_RB1	-0.0001	0	-0.0001	0.0092
cnv_CHEK1	0	0	0	0.0091
cnv_CBL	0	0	0	0.0089
wes_BRCA1	0	0	0	0.0089
cnv_FGF3	0	0	0	0.0088
wes_CHEK2	0	0	0	0.0085
wes_AXL	0	0	0	0.0082
wes_ERBB4	0	0	0	0.0082
cnv_SDHD	0	0	0	0.0078
cnv_MEN1	0	0	0	0.0077
cnv_FANCF	0	0	0	0.0066
wes_FLT3	0	0	0	0.0063
wes_SPEN	0	0	0	0.0062
cnv_HRAS	0	0	0	0.0062
cnv_FANCA	0	0	0	0.0057
wes_RPTOR	0	0	0	0.0053
cnv_MLL	0	0	0	0.0053
wes_GNAS	0	0	0	0.0052
wes_ATM	0	0	0	0.0046
wes_MTOR	0	0	0	0.0042
wes_ERBB2	0	0	0	0.0040
wes_RAD50	0	0	0	0.0039
wes_CDKN1A	0	0	0	0.0038
cnv_SMAD2	0	0	0	0.0038
wes_STK11	0	0	0	0.0038
cnv_CIC	0	0	0	0.0038
cnv_NKX2.1	0	0	0	0.0036
wes_EP300	0	0	0	0.0036
wes_PLCG2	0	0	0	0.0033
wes_SMAD4	0	0	0	0.0033
wes_TSC2	0	0	0	0.0033

wes_NOTCH3	0	0	0	0.0030
wes_PIK3CG	0	0	0	0.0030
cnv_BCL2L2	0	0	0	0.0029
wes_CDKN2A	0	0	0	0.0029
cnv_NOTCH1	0	0	0	0.0028
wes_GRIN2A	0	0	0	0.0024
cnv_SMO	0	0	0	0.0023
cnv_FGFR1	0	0	0	0.0023
cnv_FGFR4	0	0	0	0.0020
cnv_CD79A	0	0	0	0.0020
wes_CTNNA1	0	0	0	0.0013
cnv_DOT1L	0	0	0	0.0006
wes_SMARCA4	0	0	0	0.0006
cnv_FANCG	0	0	0	0.0002

APPENDIX 6: RELEVANT PUBLISHED WORK AND ABSTRACTS

ABSTRACT PRESENTED AT THE AMERICAN SOCIETY OF CLINICAL PHARMACOLOGY AND THERAPEUTICS 2017 ANNUAL MEETING, WASHINGTON, D.C.

Identifying somatic mutations associated with intrinsic resistance to multi-targeted tyrosine kinase inhibitors.

Nancy K. Gillis, Christine M. Walko, Daniel M. Rotroff, Tania E. Mesa, Sean J. Yoder, Zhihua Chen, Jamie K. Teer, Howard L. McLeod

Background: Approximately 15% - 30% of individuals with therapeutic indications for multi-targeted tyrosine kinase inhibitors (TKIs) fail to show even transitory benefit, demonstrating intrinsic resistance (IR). Early identification of inherently resistant tumors is essential to avoid exposure to non-effective therapies and unnecessary toxicities and to guide selection toward other therapies.

Methods: We conducted a retrospective cohort study to identify somatic mutations associated with IR to multi-targeted TKIs. Adult patients treated with multi-targeted TKIs were classified as intrinsically resistant (progression at 1st imaging follow-up), non-resistant (no progression), or unknown (insufficient follow-up). Targeted exome or whole exome sequencing was performed using tumor FFPE samples (data collection completed 9/7/16, giving insufficient time for analysis by the 9/8 deadline). A candidate gene approach was employed to identify differences in mutations between resistant and non-resistant individuals.

Results: A total of 51 individuals were included (median age 61 yo). The most prevalent tumor type was renal cell (48%). The majority received sorafenib (29%), sunitinib (27%), or pazopanib

(41%). Thirty percent demonstrated IR. Median time to drug discontinuation was statistically different between resistant and non-resistant individuals (2.2 vs 8.5 mo, $p < 0.001$). Five genes (NTRK1, KDR, TGFBR2, PTPN11, and NOTCH2) were more commonly mutated in resistant individuals (OR = 5.5, insufficient power). These genes have strong biologic plausibility supporting their potential roles in resistance to TKIs.

Conclusion: Somatic mutations in multi-targeted TKI drug targets may serve as biomarkers of IR to these agents. Future studies will further explore these associations.

ABSTRACT PRESENTED AT THE AMERICAN ASSOCIATION FOR CANCER RESEARCH 2016 ANNUAL MEETING, NEW ORLEANS, LA

Prevalence and triggers of drug-induced Stevens-Johnson syndrome (SJS) and toxic epidermal necrolysis (TEN) in a cancer patient cohort.

Nancy K. Gillis, Gillian C. Bell, Amy J. Brandt, Howard L. McLeod

SJS and TEN are extremely rare (approximately 2-7 cases per million per year) hypersensitivity reactions most commonly attributed to medications. The life-threatening nature of SJS/TEN necessitates early diagnosis and immediate identification and withdrawal of causative agents. Some of the most commonly associated culprits include sulfonamide antibiotics, aromatic anticonvulsants (phenytoin, carbamazepine, lamotrigine), -oxicam NSAIDs (e.g., meloxicam), allopurinol, and nevirapine. Individual case reports of SJS/TEN have been associated with anti-cancer agents, but a comprehensive assessment has not been performed. A large-scale retrospective analysis of the prevalence of SJS/TEN in cancer patients treated at Moffitt Cancer Center (MCC) between Jan 1, 2002 – Dec 31, 2014 was conducted. A total of 104,917 cancer patients were screened for SJS/TEN-related ICD-9 codes in the MCC health research informatics database. SJS/TEN diagnoses were identified in 121 patients. Manual chart review of physician, dermatology, and pathology notes confirmed SJS/TEN in 47 patients (in-patient + historical) and possible SJS/TEN in an additional 17 patients, corresponding to an overall prevalence of 0.06%. Confirmed in-patient cases of SJS/TEN were more common in hematologic malignancies compared to solid tumors (n = 12 vs. n=7, respectively). Notably, 5 of the 19 (26.3%) confirmed cases were observed in patients with acute myeloid leukemia. Physician-reported culprits for SJS/TEN diagnoses included antibiotics, immunomodulators,

anticonvulsants, and anti-cancer agents. The observed prevalence of SJS/TEN was higher in cancer patients than previous reports from the general USA population. There were 19 confirmed in-patient diagnoses of SJS/TEN and an additional 45 historical and possible cases of SJS/TEN in 104,917 cancer patients, corresponding to an overall prevalence of 0.018% to 0.06%, an approximately 90-fold increase when compared to the general population. Possible explanations for increased risk in cancer patients include increased exposure to culprit medications, cancer disease process, immunocompromised state, or synergy between risk factors. A thorough understanding of the factors that increase risk to SJS/TEN in cancer patients is critical to facilitate culprit withdrawal and maximize patient outcomes and survival.

ABSTRACT PRESENTED AT THE AMERICAN PHARMACISTS ASSOCIATION 2016 MIDYEAR MEETING, LAS VEGAS, NV

Exploring potential predictors of intrinsic resistance to multi-targeted tyrosine kinase inhibitors in patients with solid tumors

Michael A. Carulli, Christine M. Walko, Howard L. McLeod, Nancy K. Gillis

Purpose: Tyrosine kinase inhibitors (TKIs) are commonly prescribed antineoplastics used to treat a variety of cancer types. Unfortunately, approximately 15-25 percent of patients will not respond to treatment, which poses a significant burden considering the high potential for side effects and cost of these medications. This study explores the demographics and clinical factors of patients treated with multi-targeted TKIs in the context of whether they derived benefit or were resistant to treatment. We conducted a retrospective cohort study to investigate potential predictive factors of response in patients prescribed multi-targeted TKIs (i.e., axitinib, cabozantinib, pazopanib, regorafenib, sorafenib, sunitinib, or vandetanib).

Methods: Patients were identified from Moffitt Cancer Center's Total Cancer Care cohort with the inclusion criteria that they received a multi-targeted TKI and were diagnosed with a solid tumor. We performed chart reviews of electronic medical records to confirm diagnosis, TKI administration, and duration of treatment. Patient response to TKI was determined using imaging scans before TKI initiation and at first imaging follow-up, incorporating tumor size changes and radiologists' and oncologists' impressions to classify individuals as either a "responder" or "non-responder (resistant)". Patients with complete response, partial response, mixed response, or stable disease were classified as "responders", while patients who had progressive disease at first imaging follow-up were classified as "non-responders"; patients who discontinued treatment

early due to side effects were excluded. Data was organized using Microsoft Excel, and demographic information, including age at diagnosis, sex, race, ethnicity, diagnosis, and multi-targeted TKI received was summarized. Each demographic and clinical category was evaluated to determine the rate of resistance based on the number of patients who were non-responders divided by the total number of patients, in that category, who received the drug. We calculated the average, median, and range of treatment duration stratified by response, medication, and diagnosis. The primary objective of this study was to identify demographic or clinical variables that may be associated with resistance to multi-targeted TKIs.

Results: A total of 262 solid tumor patients who were prescribed multi-targeted TKIs were included in this study. The majority of patients were white (94 percent), non-Hispanic (92 percent), and the median age was 56 years old (range 21-88). The most common diagnosis in our cohort was renal cell carcinoma (RCC, 52 percent), followed by sarcoma (20 percent), and less than 10 percent for all other tumor types. Half of the patients received sunitinib, followed by sorafenib (28 percent). A total of 77 patients were excluded from further analyses due to unknown response. The overall resistance rate in our cohort was 21 percent, consistent with that observed in phase III clinical trials of these medications. Responders remained on treatment significantly longer than non-responders (median duration 11.0 mo vs. 3.6 mo, p less than 0.001). Patients aged 60-69 had a higher rate of resistance than expected (30 percent), as did patients who received pazopanib (35 percent). Tumor types with higher resistance rates than expected included melanoma (43 percent), pancreatic neuroendocrine (36 percent) and sarcoma (28 percent), among others. Overall, patients who received axitinib remained on therapy the longest (median 11.7 mo, range 9.6-20.7 mo).

Conclusion: Multi-targeted TKIs are highly effective therapies for a wide range of tumor types; however, according to phase III clinical trials, approximately 15-25 percent of patients do not respond. We conducted a retrospective cohort study to identify demographic and clinical factors that may be predictive of response to these agents. Results from our study suggest that demographic and clinical factors, including but not limited to, sex, age, and diagnosis, may influence likelihood of response to multi-targeted TKIs. Future studies will explore the utility of genetics to better predict resistance to these widely-prescribed medications.

ABSTRACT PRESENTED AT THE H. LEE MOFFITT CANCER CENTER AND RESEARCH INSTITUTE 2015 SCIENTIFIC SYMPOSIUM, TAMPA, FL

Identifying genetic predictors of intrinsic resistance to multi-targeted tyrosine kinase inhibitors

Nancy K. Gillis, Anders E. Berglund, Howard L. McLeod

Background: While results from multi-targeted tyrosine kinase inhibitor (TKI) clinical trials demonstrated overall efficacy, approximately 20% of individuals failed to show even transitory clinical benefit, demonstrating inherent resistance. Early identification of inherently resistant tumors is essential to avoid exposure of patients to non-effective therapies and unnecessary toxicities, and to guide selection of other potentially effective therapies.

Methods: This is a retrospective cohort study to identify genetic predictors of inherent resistance to TKIs across tumor types. All Moffitt Total Cancer Care (TCC) patients who received a multi-targeted TKI and have gene expression data available are included. Candidate genes were selected based on the major drug targets of the selected TKIs. This list was further expanded to include additional genes within the same pathway or genes shown to interact with the drug targets.

Results: A total of 2,511 TCC patients received a multi-targeted TKI; 144 (5.7%) have gene expression data available. There are a wide range of primary tumor sites included, with kidney (44%), colorectal (8%), and soft tissue (6%) tumors being most prevalent. Candidate gene selection identified 28 genes that are known to interact with ≥ 4 of the 7 multi-targeted TKIs. There is wide variability (>2 log2-fold) in the expression level of 23 (82%) of candidate genes.

PCA and correlation analysis indicate that many of the candidate genes show unique expression patterns across individuals.

Conclusion: We observed high variability in mRNA expression of candidate genes hypothesized to be important in the pharmacodynamics of multi-targeted TKIs. Future work will be aimed at exploiting the observed differences in expression to identify patients who will be inherently resistant to TKIs.

APPENDIX 7: GRANTS AND AWARDS

Spring 2017 **University of North Carolina Off-Campus Dissertation Fellowship**

Scholarship awarded to doctoral students conducting impressive research and making notable progress toward degree completion while away from the UNC campus

2016 – 2017 **American Foundation for Pharmaceutical Education Pre-Doctoral Fellowship in Pharmaceutical Sciences**

National award given to doctoral students who possess the skill and aptitude to become outstanding scientists and leaders in the pharmaceutical industry, academia, and the government/nonprofit sectors

Fall 2016 **American Society of Hematology Abstract Achievement Award**

Merit-based scholarship awarded to top abstracts selected for presentation at the ASH annual conference

Spring 2016 **Khalid Ishaq Graduate Travel Award**

Awarded to graduate students presenting research at national conferences

2012 – 2013 **Eshelman Fellowship Award**

Merit-based award for selected incoming students into the graduate program at UNC

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